



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07D 221/02, 215/12, C12Q 1/70, 1/68, 1/08, C12P 21/06, C12N 15/00, 9/14, 9/84, 9/86, G01N 33/53, C07H 21/02, 21/04, A01N 43/04		A1	(11) International Publication Number: WO 98/13353 (43) International Publication Date: 2 April 1998 (02.04.98)
<p>(21) International Application Number: PCT/US97/17395</p> <p>(22) International Filing Date: 26 September 1997 (26.09.97)</p> <p>(30) Priority Data: 08/719,697 26 September 1996 (26.09.96) US</p> <p>(71) Applicant (<i>for all designated States except US</i>): AURORA BIOSCIENCES CORPORATION [US/US]; 11149 N. Torrey Pines Road, La Jolla, CA 92037 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): WHITNEY, Michael, A. [US/US]; 8320 Via Sonoma #81, La Jolla, CA 92037 (US). NEGULESCU, Paul, A. [US/US]; 678 Solana Circle, Solana Beach, CA 92075 (US). CRAIG, Frank [US/US]; 409 Santa Helena, Solana Beach, CA 92075 (US). MERE, Lora [US/US]; 3522 Syracuse Avenue, San Diego, CA 92122 (US). FOULKES, Gordon, J. [US/US]; 1220 Rancho Encinitas Drive, Encinitas, CA 92024 (US).</p> <p>(74) Agent: HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(54) Title: METHODS AND COMPOSITIONS FOR SENSITIVE AND RAPID, FUNCTIONAL IDENTIFICATION OF GENOMIC POLYNUCLEOTIDES AND USE FOR CELLULAR ASSAYS IN DRUG DISCOVERY</p> <p>(57) Abstract</p> <p>The invention provides for methods and compositions for identifying proteins or chemicals that directly or indirectly modulate a genomic polynucleotide and methods for identifying active genomic polynucleotides. Generally, the method comprises inserting a BL (beta-lactamase) expression construct into an eukaryotic genome, usually non-yeast, contained in at least one living cell, contacting the cell with a predetermined concentration of a modulator, and detecting BL activity in the cell.</p>	

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Methods and Compositions for Sensitive and Rapid, Functional Identification of Genomic Polynucleotides and Use For Cellular Assays in Drug Discovery

5

Cross Reference to Related Applications

This application claims the benefit of an earlier filing date to a patent application identified as United States patent application No.: 08/719,697, filed September 26, 1996,
10 entitled "Methods and compositions for sensitive and rapid, functional identification of genomic polynucleotides and secondary screening capabilities" of which the present application as a Continuation-in-Part and which is incorporated herein by reference.

Technical Field

15 The present invention generally relates to methods and compositions for the identification of useful and functional portions of the genome and compounds for modulating such portions of the genome, particularly the identification of proteins that are directly or indirectly transcriptionally regulated and compounds for regulating such proteins, either directly or indirectly.

20

Background

The identification and isolation of useful portions of the genome requires extensive expenditure of time and financial resources. Currently, many genome projects use various strategies to reduce cloning and sequencing times. While genome projects
25 rapidly expand the database of genetic material, such projects often lack the ability to integrate the information with the biology of the cell or organism from which the genes were isolated. In some instances, coding regions of newly isolated genes reveal sequence homology to other genes of known function. This type of analysis can, at best, provide clues as to the possible relationships between different genes and proteins. Genomic
30 projects in general, however, suffer from the inability to rapidly and directly isolate, and

identify specific, yet unknown, genes associated with particular a biological process or processes.

The evaluation of the function of genes identified from genomic sequencing projects requires cloning the discovered gene into an expression system suitable for functional screening. Transferring the discovered gene into a functional screening system requires additional expenditure of time and resources without a guarantee that the correct screening system was chosen. Since the function of the discovered gene is often unknown or only surmised by inference to structurally related genes, the chosen screening system may not have any relationship to the biological function of the gene.

For example a gene may encode a protein that is structurally homologous to the beta-adrenergic receptor and have a dissimilar function. Further, if negative results are obtained in the screen, it can not be easily determined whether 1) the gene or gene product is not functioning properly in the screening assay or 2) the gene or gene product is directly or indirectly involved in the biological process being assayed by the screening system.

Consequently, there is a need to provide methods and compositions for rapidly isolating portions of genomes associated with a known biological process and to screen such portions of genomes for activity without the necessity of transferring the gene of interest into an additional screening system.

20

Brief Description of the Figures

FIG. 1 shows a comparison between an application of a prior art reporter gene with methods described herein, and one embodiment of the invention. The prior art uses the b-gal reporter and requires the establishment of clones prior to expression analysis.

One embodiment of this invention allows for the rapid identification of living cell clones from large multiclonal populations of BLEC (beta-lactamase expression construct) integrated cells. This is a significant advancement over the prior art, which requires the analysis of individual clones followed by the retrieving of selected clone from a duplicate clonal stock of living cells.

FIG. 2 shows a representation of how one embodiment of the invention reports the expression of a pathway within a cell and can be used for screening.

FIG. 3 shows a schematic plasmid map of the BLEC-1.

FIG. 4 shows the FACS analysis of a population of genetically BLEC integrated clones. Individually cells are plotted by fluorescent emission properties at 400 nm excitation. The x axis represents green emission (530 nm). The y axis represents blue emission (465 nm). Cells with a high blue/green ration will appear blue in color and cells with a low blue/green ratio will appear green in color. A) Unselected multiclonal population of BLEC integrated RBL-1 cell clones. B) Population of clones sorted from 3A (R1) that were cultured for an additional 7 days and resorted. C) Population from 3B with addition of 1uM ionomycin for 12 hours prior to sorting.

Summary

The present invention recognizes that β -lactamase polynucleotides can be effectively used in living eukaryotic cells to functionally identify active portions of a genome directly or indirectly associated with a biological process. The present invention also recognizes for the first time that β -lactamase activity can be measured using membrane permeant substrates in living cells incubated with a test chemical that directly or indirectly interacts with a portion of the genome having an integrated β -lactamase polynucleotide. The present invention, thus, permits the rapid identification and isolation of genomic polynucleotides indirectly or directly associated with a defined biological process and identification of compounds that modulate such processes and regions of the genome. Because the identification of active genomic polynucleotides is permitted in living cells, further functional characterization can be conducted using the same cells, and optionally, the same screening assay. The ability to functionally screen immediately after the rapid identification of a functionally active portion of a genome, without the necessity of transferring the identified portion of the genome into a secondary screening system, represents, among other things, a distinct advantage over an application of a prior art reporter gene with the methods described herein, as shown in **FIG. 1**.

The invention provides for a method of identifying portions of a genome, e.g. genomic polynucleotides, in a living cell using a polynucleotide encoding a protein with β -lactamase activity that can be detected with a membrane permeant β -lactamase substrate. Typically, the method involves inserting a polynucleotide encoding a protein with β -lactamase activity into the genome of an organism using any method known in the art, developed in the future or described herein. Usually, a β -lactamase expression construct will be used into integrate a β -lactamase polynucleotide into a eukaryotic genome, as described herein. The cell, such as a eukaryotic cell, is usually contacted with a predetermined concentration of a modulator, either before or after integration of the β -lactamase polynucleotide. β -lactamase activity is usually then measured inside the living cell, preferably with fluorescent, membrane permeant β -lactamase substrates that are transformed by the cell into membrane impermeant β -lactamase substrates as described herein.

The invention also provides for a method of identifying proteins or compounds that directly or indirectly modulate a genomic polynucleotide. Generally, the method comprises inserting a β -lactamase expression construct into an eukaryotic genome, usually non-yeast, contained in at least one living cell, contacting the cell with a predetermined concentration of a modulator, and detecting β -lactamase activity in the cell.

The invention also provides for a method of screening compounds with an active genomic polynucleotide that comprises: 1) optionally contacting a multiclonal population of cells with a first test chemical prior to separating said cells by a FACS, 2) separating by a FACS said multiclonal population of cells into β -lactamase expressing cells and non β -lactamase expressing cells, wherein said β -lactamase expressing cells have a detectable difference in cellular fluorescence properties compared to non- β -lactamase expressing cells, 3) contacting either population of cells with the same or a different test chemical, and 4) optionally repeating step (2), wherein said multi-clonal population of cells comprises eukaryotic cells having a β -lactamase expression construct integrated into a genome of said eukaryotic cells and a membrane permanent β -lactamase substrate

transformed inside said cells to a membrane impermeant β -lactamase substrate. The steps of this method can be repeated to permit additional characterization of identified clones.

The invention also includes powerful methods and compositions for identifying 5 physiologically relevant cellular pathways and proteins of interest of known, unknown or partially known function. As shown in FIG. 2 a pathway may have more than one major intracellular signal. Two major intracellular pathways are shown ("A" and "B"). Each intracellular signal pathway may also have multiple branches. Each arm is shown as having three signaling pathways (A1, A2, and A3; and B1, B2, and B3). By generating a 10 library of clones with a β -lactamase expression construct, genomic polynucleotides for each signal pathway can be tagged or reported by the expression of β -lactamase.

Pathways not effected by the modulator (shown as C1, C2, and C3) are also tagged with 15 β -lactamase expression construct. Because the modulator only modulates the expression of pathways A1, A2, A3, B1, B2, and B3, only clones corresponding to these genomic integration sites are identified as being responsive to the modulator. Clones corresponding to sites C1, C2, and C3 remain unaltered and are not responsive to the modulator. Any individual, modulated clone can be immediately isolated, if not already isolated, and used for a drug discovery assay to screen test chemicals for activity for modulating the reported pathway, as described herein.

20 The invention also includes tools for pathway identification and drug discovery that can be applied to a number of targets of interest and therapeutic areas including, proteins of interest, physiological responses even in the absence of a definitive target (e.g. immune response, signal transduction, neuronal function and endocrine function), viral targets, and orphan proteins.

Detailed Description of the Invention

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection).

Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, organic synthetic chemistry, and pharmaceutical formulation described below are those well known and commonly employed in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical formulation and delivery, and treatment of patients. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

"Fluorescent donor moiety" refers to a fluorogenic compound or part of a compound (including a radical) which can absorb energy and is capable of transferring the energy to another fluorogenic molecule or part of a compound. Suitable donor fluorogenic molecules include, but are not limited to, coumarins and related dyes xanthene dyes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanine dyes, bimanes, acridines, isoindoles, dansyl dyes, aminophthalic hydrazides such as luminol and isoluminol derivatives, aminophthalimides, aminonaphthalimides,

aminobenzofurans, aminoquinolines, dicyanohydroquinones, and europium and terbium complexes and related compounds.

"Quencher" refers to a chromophoric molecule or part of a compound that is capable of reducing the emission from a fluorescent donor when attached to the donor.

5 Quenching may occur by any of several mechanisms including fluorescence resonance energy transfer, photoinduced electron transfer, paramagnetic enhancement of intersystem crossing, Dexter exchange coupling, and exciton coupling such as the formation of dark complexes.

"Acceptor" refers to a quencher that operates via fluorescence resonance energy transfer. Many acceptors can re-emit the transferred energy as fluorescence. Examples include coumarins and related fluorophores, xanthenes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanines, difluoroboradiazaindacenes, and phthalocyanines. Other chemical classes of acceptors generally do not re-emit the transferred energy. Examples include indigos, benzoquinones, anthraquinones, azo compounds, nitro compounds, indoanilines, di- and triphenylmethanes.

15 "Dye" refers to a molecule or part of a compound that absorbs specific frequencies of light, including but not limited to ultraviolet light. The terms "dye" and "chromophore" are synonymous.

"Fluorophore" refers to a chromophore that fluoresces.

20 "Membrane-permeant derivative" refers a chemical derivative of a compound of that increases membrane permeability of the compound. These derivatives are made better able to cross cell membranes, i.e. membrane permeant, because hydrophilic groups are masked to provide more hydrophobic derivatives. Also, the masking groups are designed to be cleaved from the fluorogenic substrate within the cell to generate the 25 derived substrate intracellularly. Because the substrate is more hydrophilic than the membrane permeant derivative it is now trapped within the cells.

"Isolated polynucleotide" refers to a polynucleotide of genomic, cDNA, or synthetic origin or some combination there of, which by virtue of its origin the "isolated polynucleotide" (1) is not associated with the cell in which the "isolated polynucleotide"

is found in nature, or (2) is operably linked to a polynucleotide which it is not linked to in nature.

"Isolated protein" refers to a protein of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated protein" (1) 5 is not associated with proteins found it is normally found with in nature, or (2) is isolated from the cell in which it normally occurs or (3) is isolated free of other proteins from the same cellular source, e.g. free of human proteins, or (4) is expressed by a cell from a different species, or (5) does not occur in nature.

"Polypeptide" as used herein as a generic term to refer to native protein, 10 fragments, or analogs of a polypeptide sequence. Hence, native protein, fragments, and analogs are species of the polypeptide genus. Preferred, β -lactamase polypeptides include those with the polypeptide sequence represented in the **SEQUENCE ID**.

LISTING and any other polypeptide or protein having similar β -lactamase activity as measured by one or more of the assays described herein. β -lactamase polypeptide or 15 proteins can include any protein having sufficient activity for detection in the assays described herein.

"Naturally-occurring" as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in 20 nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression 25 of the coding sequence is achieved under conditions compatible with the control sequences.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding and non-coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in

prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA. "Genomic polynucleotide" refers to a portion of a genome. "Active genomic polynucleotide" or "active portion of a genome" refer to regions of a genome that can be up regulated, down-regulated or both, either directly or indirectly, by a biological process. "Directly," in the context of a biological process or processes, refers to direct causation of a process that does not require intermediate steps, usually caused by one molecule contacting or binding to another molecule (the same type or different type of molecule). For example, molecule A contacts molecule B, which causes molecule B to exert effect X that is part of a biological process. "Indirectly," in the context of a biological process or processes, refers to indirect causation that requires intermediate steps, usually caused by two or more direct steps. For example, molecule A contacts molecule B to exert effect X which in turn causes effect Y.

" β -lactamase polynucleotide" refers to a polynucleotide encoding a protein with β -lactamase activity. Preferably, the protein with β -lactamase activity can measured be in a FACS at 22°degrees using a CCF2-AM β -lactamase substrate at a level of about 1,000 such protein molecules or less per cell. More preferably, the protein with β -lactamase activity can measured be in a FACS at 22° degrees using a CCF2-AM β -lactamase substrate at a level of about 300 to 1,000 such protein molecules per cell. More preferably, the protein with β -lactamase activity can measured be in a FACS at 22° degrees using a CCF2-AM β -lactamasesubstrate at a level of about 25 to 300 such protein molecules per cell. Proteins with β -lactamaseactivity that require more than 1,000

molecules of such protein per cell for detection with a FACS at 22° degrees using a CCF2-AM β-lactamase substrate can be used and preferably have at least 5% of the activity of the protein with SEQ. ID. NO.: 1.

"Sequence homology" refers to the proportion of base matches between two nucleic acid sequences or the proportion amino acid matches between two amino acid sequences. When sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of sequence from a desired sequence (e.g. β-lactamase sequences, such as SEQ. ID. NO.: 1) that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less are preferred with 2 bases or less more preferred. When using oligonucleotides as probes or treatments the sequence homology between the target nucleic acid and the oligonucleotide sequence is generally not less than 17 target base matches out of 20 possible oligonucleotide base pair matches (85%); preferably not less than 9 matches out of 10 possible base pair matches (90%), and most preferably not less than 19 matches out of 20 possible base pair matches (95%).

"Selectively hybridize" refers to detectably and specifically bind. Polynucleotides, oligonucleotides and fragments thereof selectively hybridize to target nucleic acid strands, under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. High stringency conditions can be used to achieve selective hybridization conditions as known in the art and discussed herein. Generally, the nucleic acid sequence homology between the polynucleotides, oligonucleotides, and fragments thereof and a nucleic acid sequence of interest will be at least 30%, and more typically with preferably increasing homologies of at least about 40%, 50%, 60%, 70%, and 90%.

Typically, hybridization and washing conditions are performed at high stringency according to conventional hybridization procedures. Positive clones are isolated and sequenced. For illustration and not for limitation, a full-length polynucleotide corresponding to the nucleic acid sequence of SEQ. ID.NO. 1 may be labeled and used as

a hybridization probe to isolate genomic clones from a the appropriate target library in λ EMBL4 or λ GEM11 (Promega Corporation, Madison, Wisconsin); typical hybridization conditions for screening plaque lifts (Benton and Davis (1978) Science 196: 180) can be: 50% formamide, 5 x SSC or SSPE, 1-5 x Denhardt's solution, 0.1-1% SDS, 5 100-200 μ g sheared heterologous DNA or tRNA, 0-10% dextran sulfate, 1×10^5 to 1×10^7 cpm/ml of denatured probe with a specific activity of about 1×10^8 cpm/ μ g, and incubation at 42°C for about 6-36 hours. Prehybridization conditions are essentially identical except that probe is not included and incubation time is typically reduced. Washing conditions are typically 1-3 x SSC, 0.1-1% SDS, 50-70°C with change of wash 10 solution at about 5-30 minutes. Cognate sequences, including allelic sequences, can be obtained in this manner.

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. 15 Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) 20 using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M.O., in Atlas of Protein Sequence and Structure, 1972, volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 30% identical when optimally aligned using 25 the ALIGN program.

"Corresponds to" refers to a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to all or a portion of a reference polypeptide sequence. In contradistinction, the term "complementary to" is

used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

5 The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity." A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length
10 cDNA or gene sequence given in a sequence listing such as a SEQ. ID. NO.: 1, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two
15 polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20
20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal
25 alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP,

BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" 5 means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched 10 positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide 15 comprises a sequence that has at least 30 percent sequence identity, preferably at least 50 to 60 percent sequence identity, more usually at least 60 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the 20 polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 30 percent sequence identity, preferably at least 40 percent sequence identity, more preferably at least 50 percent sequence identity, and most 25 preferably at least 60 percent sequence identity. Preferably, residue positions, which are not identical, differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is

serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamic-aspartic, and asparagine-glutamine.

"Polypeptide fragment" refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is usually identical to the corresponding positions in the naturally-occurring sequence deduced, for example, from a full-length cDNA sequence (e.g., the sequence shown in SEQ. ID. NO.: 1). "β-lactamase polypeptides fragment" refers to a polypeptide that is comprised of a segment of at least 25 amino acids that has substantial identity to a portion of the deduced amino acid sequence shown in SEQ. ID. NO.:1 and which has at least one of the following properties: (1) specific binding to a β-lactamase substrate, preferably cephalosporin, under suitable binding conditions, or (2) the ability to effectuate enzymatic activity, preferably cephalosporin backbone cleavage activity, when expressed in a mammalian cell. Typically, analog polypeptides comprise a conservative amino acid substitution (or addition or deletion) with respect to the naturally occurring sequence. 20 Analogs typically are at least 300 amino acids long, preferably at least 500 amino acids long or longer, most usually being as long as full-length naturally-occurring polypeptide.

"Modulation" refers to the capacity to either enhance or inhibit a functional property of a biological activity or process (e.g., enzyme activity or receptor binding). Such enhancement or inhibition may be contingent on the occurrence of a specific event, 25 such as activation of a signal transduction pathway, and/or may be manifest only in particular cell types.

The term "modulator" refers to a chemical (naturally occurring or non-naturally occurring), such as a biological macromolecule (e.g. nucleic acid, protein, non-peptide, or organic molecule), or an extract made from biological materials such as bacteria, plants,

fungi, or animal (particularly mammalian) cells or tissues. Modulators are typically evaluated for potential activity as inhibitors or activators (directly or indirectly) of a biological process or processes (e.g., agonist, partial antagonist, partial agonist, antagonist, antineoplastic agents, cytotoxic agents, inhibitors of neoplastic transformation or cell proliferation, cell proliferation-promoting agents, and the like) by inclusion in assays described herein. The activity of a modulator may be known, unknown or partial known.

The term "test chemical" refers to a chemical to be tested by one or more method(s) of the invention as a putative modulator. A test chemical is usually not known to bind to the target of interest. The term "control test chemical" refers to a chemical known to bind to the target (e.g., a known agonist, antagonist, partial agonist or inverse agonist). The term "test chemical" does not typically include a chemical added as a control condition that alters the function of the target to determine signal specificity in an assay. Such control chemicals or conditions include chemicals that 1) non-specifically or substantially disrupt protein structure (e.g., denaturing agents (e.g., urea or guandium), charotropic agents, sulphydryl reagents (e.g., dithiotritol and β -mercaptoethanol), and proteases), 2) generally inhibit cell metabolism (e.g., mitochondrial uncouplers) and 3) non-specifically disrupt electrostatic or hydrophobic interactions of a protein (e.g., high salt concentrations, or detergents at concentrations sufficient to non-specifically disrupt hydrophobic interactions). The term "test chemical" also does not typically include chemicals known to be unsuitable for a therapeutic use for a particular indication due to toxicity of the subject. Usually, various predetermined concentrations test chemicals are used for screening such as .01 μ M, .1 μ M, 1.0 μ M, and 10.0 μ M.

The term "target" refers to a biochemical entity involved a biological process. Targets are typically proteins that play a useful role in the physiology or biology of an organism. A therapeutic chemical binds to target to alter or modulate its function. As used herein, targets can include cell surface receptors, G-proteins, kinases, ion channels, phospholipases and other proteins mentioned herein.

- The terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods).
- 5 Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g., ^3H , ^{14}C , ^{35}S , ^{125}I , ^{131}I), fluorescent labels (e.g., FITC, rhodamine, and lanthanide phosphors), enzymatic labels (or reporter genes) (e.g., enzymatic reporter genes horseradish peroxidase, β -galactosidase, luciferase and alkaline 10 phosphatase; and non-enzymatic reporter genes (e.g., fluorescent proteins)), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). "Substantially pure" refers to an object species is the predominant species present (i.e., on a molar basis it is more abundant than 15 any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, more preferably more than about 85%, 90%, 95%, and 99%. 20 Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.
- "Pharmaceutical agent or drug" refers to a chemical or composition capable of inducing a desired therapeutic effect when properly administered (e.g. using the proper 25 amount and delivery modality) to a patient.
- Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco, incorporated herein by reference).

Introduction

The present invention recognizes that β -lactamase polynucleotides can be effectively used in living eukaryotic cells to functionally identify active portions of a genome directly or indirectly associated with a biological process. The present invention 5 also recognizes for the first time that β -lactamase activity can be measured using membrane permeant substrates in living cells incubated with a test chemical that directly or indirectly interacts with a portion of the genome having an integrated β -lactamase polynucleotide. The present invention, thus, permits the rapid identification and isolation of genomic polynucleotides indirectly or directly associated with a defined 10 biological process and identification of compounds that modulate such processes and regions of the genome. Because the identification of active genomic polynucleotides is permitted in living cells, further functional characterization can be conducted using the same cells, and optionally, the same screening assay. The ability to functionally screen immediately after the rapid identification of a functionally active portion of a genome, 15 without the necessity of transferring the identified portion of the genome into a secondary screening system, represents, among other things, a distinct advantage over an application of a prior art reporter gene and methods described herein, as shown in FIG. 1.

As a non-limiting introduction to the breadth of the invention, the invention includes several general and useful aspects, including:

- 20 1) a method for identifying genes or gene products directly or indirectly associated (e.g. regulated) with a biological process of interest (that can be modulated by a compound) using a genomic polynucleotide operably linked to a polynucleotide encoding a protein with β -lactamase activity,
- 2) a method for identifying proteins (e.g. orphan proteins or known proteins) or compounds that directly or indirectly modulate (e.g. activate or inhibit transcription) a genomic polynucleotide operably linked to a polynucleotide encoding a protein with β -lactamase activity,
- 25 3) a method of screening for an active genomic polynucleotide (e.g. enhancer, promoter or coding region in the genome) that can be directly or indirectly

associated (e.g. regulated) with a biological process of interest (that can be modulated by a compound) using a genomic polynucleotide operably linked to a polynucleotide encoding a protein with β -lactamase activity that can be detected by FACS using a fluorescent, membrane permeant β -lactamase substrate,

- 5 4) eukaryotic cells with a genomic polynucleotide operably linked to a polynucleotide encoding a protein with β -lactamase activity, and
5) polynucleotides related to the above methods and cells.

These aspects of the invention, as well as others described herein, can be achieved by using the methods 10 and compositions of matter described herein. To gain a full appreciation of the scope of the invention, it will be further recognized that various aspects of the invention can be combined to make desirable embodiments of the invention. For example, the invention includes a method of identifying compounds that modulate active genomic polynucleotides operably linked to a protein with β -lactamase activity that can be detected by FACS using a fluorescent, membrane permeant β -lactamase substrate. Such 15 combinations result in particularly useful and robust embodiments of the invention.

Methods for Rapidly Identifying Functional Portions of a Genome

The invention provides for a method of identifying portions of a genome, e.g. 20 genomic polynucleotides, in a living cell using a polynucleotide encoding a protein with β -lactamase activity that can be detected with a membrane permeant β -lactamase substrate. Typically, the method involves inserting a polynucleotide encoding a protein 25 with β -lactamase activity into the genome of an organism using any method known in the art, developed in the future or described herein. Usually, a β -lactamase expression construct will be used to integrate a β -lactamase polynucleotide into a eukaryotic genome, as described herein. The cell, such as a eukaryotic cell, is usually contacted with a predetermined concentration of a modulator, either before or after integration of the β -lactamase polynucleotide. β -lactamase activity is usually then measured inside the living cell, preferably with fluorescent, membrane permeant β -lactamase substrates that are transformed by the cell into membrane impermeant β -lactamase substrates as described herein and PCT Publication No. WO96/30540 published October 3, 1996, by Tsien et al.

Once β -lactamase polynucleotides are integrated into the genome of interest, they become under the transcriptional control of the genome of the host cell. Integration into the genome is usually stable, as described herein and known in the art. Transcriptional control of the genome often results from receptor (e.g. intracellular or cell surface receptor) activation, which can regulate transcriptional and translational events to change the amount of protein present in the cell. The amount of protein present with β -lactamase activity can be measured via its enzymatic action on a substrate. Normally, the substrate is a small uncharged molecule that, when added to the extracellular solution, can penetrate the plasma membrane to encounter the enzyme. A charged molecule can also be employed, but the charges are generally masked by groups that will be cleaved by endogenous or heterologous cellular enzymes or processes (e.g., esters cleaved by cytoplasmic esterases). As described more fully herein and in PCT Publication No. WO96/30540 published October 3, 1996, by Tsien et al., which is herein incorporated by reference, the use of substrates that exhibit changes in their fluorescence spectra upon interaction with an enzyme are particularly desirable. In some assays, the fluorogenic substrate is converted to a fluorescent product by β -lactamase activity. Alternatively, the fluorescent substrate changes fluorescence properties upon conversion by β -lactamase activity. Preferably, the product should be very fluorescent to obtain a maximal signal, and very polar, to stay trapped inside the cell.

20 *Vectors and Integration*

Vectors, such as viral and plasmid vectors, can be used to introduce genes or genetic material of the invention into cells, preferably by integration into the host cell genome. Such viral vectors can be any appropriate viruses, such as retroviruses, adenoviruses, adeno-associated viruses, papillomaviruses, herpes viruses, or any 25 ecotropic or amphotropic virus, preferably a retrovirus. The viruses can be, for example, retroviruses or any other virus modified to be replicatively deficient, cytomegalovirus, Friend leukemia virus, SIV, HIV, Rouse Sarcoma Virus, or Maloney virus such as Moloney murine leukemia virus.

Vectors, such as retrovirus vectors, can encode an operable selective protein so that cells that have been transformed can be positively selected for. Such selective protein can be antibiotic resistance factors, such as neomycin resistance, such as NEO.

Alternatively, cells can be negatively selected for using an enzyme, such as herpes simplex virus thymidine kinase (HSVTK) that transforms a pro-toxin into a toxin. Viral vectors, such as retroviral vectors, are available that are suitable for these purposes, such as PSIR vector (available from ClonTech of California with PT67 packaging cells) GgU3Hisen and GgTNKneoU3 and GgTKNeoen variants of Moloney murine leukemia virus, are available. Vector modifications can be made that allow more efficient integration into the host cell genome. Such modifications include sequences that enhance integration or known methods to promote nucleic acid transportation into the nucleus of the host cell. Retro-viral vectors are those described in U.S. Patent Number 5,364,783 by Ruley and von Melchner can also be used to increase transfection efficiency.

Vectors can also be used with liposomes or other vesicles that can transport genetic material into a cell. Appropriate structures are known in the art. The liposomes can include vectors such as plasmids or yeast artificial chromosomes (YACs), which can include genetic material to be introduced into the cell. Plasmids can also be introduced into cells by any known methods, such as electroporation, calcium phosphate, or lipofection. DNA fragments, without a plasmid or viral vector can also be used.

In one aspect of the present invention, vectors are used to introduce reporter genes into cells. When the reporter gene integrates into the genome of a target cell so that the reporter gene is expressed, that event can be detected by detecting the reporter gene. Clones that express the reporter gene under a wide variety of conditions can be used for a variety of purposes, including gene and drug discovery. Chromosomes tagged with β -lactamase expression constructs can be transferred to desired receipt cells using methods established in the art.

β -lactamase polynucleotides can be placed on a variety of plasmids for integration into a genome and to identify genes from a large variety of organisms (Gorman, C.M. et al., Mol. Cell Biol. 2: 1044-1051 (1982); Alam, J. and Cook, J.L., Anal.Biochem. 188:

245-254, (1990)). Standard techniques are used to introduce these polynucleotides into a cell or whole organism (e.g., as described in Sambrook, J., Fritsch, E.F. and Maniatis, T. Expression of cloned genes in cultured mammalian cells. In: Molecular Cloning, edited by Nolan, C. New York: Cold Spring Harbor Laboratory Press, 1989). Resistance 5 markers can be used to select for successfully transfected cells.

If a β -lactamase expression construct is selected for integrating a β -lactamase polynucleotide into a eukaryotic genome, it will usually contain at least a β -lactamase polynucleotide operably linked to a splice acceptor and optionally a splice donor.

Alternatively, the β -lactamase polynucleotide may be operably linked to any means for 10 integrating a polynucleotide into a genome, preferably for integration into an intron of a gene to produce an in frame translation product. The β -lactamase expression construct can optionally comprise, depending on the application, an IRES element, a splice donor, a poly A site, translational start site (e.g. a Kozak sequence) an LTR (long terminal repeat) and a selectable marker.

15 *β -Lactamase Reporter Genes*

Preferably, β -lactamase polynucleotides encode a cytosolic form of a protein with β -lactamase activity. This provides the advantage of trapping the normally secreted β -lactamase protein within the cell, which enhances signal to noise ratio of the signal associated with β -lactamase activity. Usually, this is accomplished by removing or 20 disabling the signal sequence normally present for secretion. As used herein, "cytosolic protein with β -lactamase activity" refers to a protein with β -lactamase activity that lacks the proper amino acid sequences for secretion from the cell, e.g., the signal sequence. For example, in the polypeptide of SEQ. ID NO.: 1, the signal sequence has been replaced with the amino acids Met-Ser. Accordingly, upon expression, β -lactamase activity 25 remains within the cell. For expression in mammalian cells it is preferably to use β -lactamase polynucleotides with nucleotide sequences preferred by mammalian cells. In some instances, a secreted form of β -lactamase can be used with the methods and compositions of the invention. In particular, genes having sequences that direct selection

can be identified with a (word) β -lactamase assay. This also permits multiplying based on directed localization of β -lactamase.

Proteins with β -lactamase activity can be any known to the art, developed in the future or described herein. This includes, for example, the enzymes represented by SEQ.

5 ID. NO.'s described herein. Nucleic acids encoding proteins with β -lactamase activity can be obtained by methods known in the art, for example, by polymerase chain reaction of cDNA using primers based on the DNA sequence in SEQ. ID. NO.: 1. PCR methods are described in, for example, U.S. Pat. No. 4,683,195; Mullis et. al. (1987) *Cold Spring Harbor Symp. Quant. Biol.* 51:263; and Erlich, ed., *PCR Technology*, (Stockton Press, 10 NY, 1989).

Sequences for Assisting Integration

The β -lactamase expression construct typically includes sequences for integration, especially sequences designed to target or enhance integration into the genome.

15 The splice site acceptor can be operably linked to the β -lactamase polynucleotide to facilitate expression upon integration into an intron. Usually, a fusion RNA will be created with the coding region of an adjacent operably portion of the exon. A splice acceptor sequence is a sequence at the 3' end of an intron where it junctions with an exon. The consensus sequences for a splice acceptor is NTN (TC) (TC) (TC) TTT (TC) (TC)(TC) (TC) (TC) NCAGgt. The intronic sequences are represented by upper 20 case and the exonic sequence by lower case font. These sequences represent those that are conserved from viral to primate genomes.

25 The splice donor site can be operably linked to the β -lactamase polynucleotide to facilitate integration in an intron to promote expression by requiring a polyadenylation sequence. Usually, a fusion RNA is created with the coding region or untranslated on the 3' end of the β -lactamase polynucleotide. This is preferred when it is desired to sequence the coding region of the identified gene. A splice donor is a sequence at the 5' end of an intron where it junctions with an exon. The consensus sequence for a splice donor sequence is naggt(ag)aGT. The intronic sequences are represented by upper case and the exonic sequence by lower case font. These sequences represent those that are conserved

from viral to primate genomes. This splice donor allows identification of the target gene using 3' RACE.

As an alternative to a splice donor site, a poly A site may be operably linked to the β -lactamase polynucleotide. Poly-adenylation signals, i.e poly A sites, include SV40 5 poly A sites, such as those described in the Invitrogen Catalog 1996 (California).

In some instances, it may be desirable to include in the β -lactamase expression construct a translational start site. For instance, a translational start site allows for β -lactamase expression even if the integration occurs in non-coding regions. Usually, such sequences will not reduce the expression of a highly expressed gene. Translational start sites 10 include a "Kozak sequence" and are the preferred sequences for expression in mammalian cells described in Kozak, M., *J. Cell Biol.* 108: 229-241 (1989). The nucleotide sequence for a cytosolic protein with β -lactamase activity in SEQ. ID. NO.: 3 contains a Kozak sequences for the nucleotides -9 to 4 (GGTACCAACCATGA).

It is also preferable, when using mammalian cells, to include an IRES ("internal 15 ribosome entry binding site") element in the β -lactamase expression construct. Typically, an IRES element will improve the yield of expressing clones. One caveat of integration vectors is that only one in three insertions into an intron will be in frame and produce a functional reporter protein. This limitation can be reduced by cloning an IRES sequence between the splice acceptor site and the reporter gene (e.g., a β -lactamase 20 polynucleotide). This eliminates reading frame restrictions and possible functional inactivation of the reporter protein by fusion to an endogenous protein. IRES elements include those from piconaviruses, picorna-related viruses, and hepatitis A and C.

Preferably, the IRES element is from a poliovirus. Specific IRES elements can be found, for instance, in WO9611211 by Das and Coward published 4/16/96, EP 585983 by Zurr 25 published 3/7/96, WO9601324 by Berlioz published 1/18/96 and WO9424301 by Smith published October 27, 1994, all of which are herein incorporated by reference.

To improve selection of β -lactamase polynucleotide into a genome, a selectable marker can be used in the β -lactamase expression construct. Selectable markers for mammalian cells are known in the art, and include for example, thymidine kinase, dihydrofolate

reductase (together with methotrexate as a DHFR amplifier), aminoglycoside phosphotransferase, hygromycin B phosphotransferase, asparagine synthetase, adenosine deaminase, metallothionein, and antibiotic resistant genes such as genes neomycin resistance. Selectable markers for non-mammalian cells are known in the art and include 5 genes providing resistance to antibiotics, such as kanamycin, tetracycline, and ampicillin.

The invention can be readily practiced with genomes having intron/exon structures. Such genomes include those of mammals (e.g., human, rabbit, mouse, rat, monkey, pig and cow), vertebrates, insects and yeast. Intron-targeted vectors are more commonly used in mammalian cells as introns, or intervening sequences, are 10 considerably larger than exons, or mRNA coding regions in mammals. Intron targeting can be achieved by cloning a splice acceptor or 3' intronic sequences upstream of a β -lactamase polynucleotide gene followed by a polyadenylation signal or 5' intronic splice donor site. When the vector inserts into an intron, the reporter gene (e.g., β -lactamase) is expressed under the same control as the gene into which it has inserted.

15 The invention can also be practiced with genomes having reduced numbers of, or lacking, intron/exon structures. For lower eukaryotes, which have simple genomic organization, i.e. containing few and small introns, exon-targeted vectors can be used. Such vectors include β -lactamase polynucleotides operably linked to a polyadenylation sequence and optionally to an IRES element. Lower eukaryotes include yeast, and fungi 20 and pathogenic eurokaryotes (e.g. parasites and microorganisms). For genomes lacking intron/exon structures restriction enzyme integration, transposon induced integration or selection integration can be used for genomic integration. Such methods include those described by Kuspa and Loomis, PNAS 89: 8803-8807 (1992) and Derbyshire, K.M., Gene Nov. 7: 143-144 (1995). Prokaryotes can be used with the invention if integration 25 can occur in such genomes. Retroviral vectors can also be used to integrate β -lactamase polynucleotides into a genome (e.g., eukaryotic), such as those methods and composition described in U.S. Patent Number 5,364,783.

Typically, integration will occur in the regions of the genome that are accessible to the integration vector. Such regions are usually active portions of the genome where

there is increased genome regulatory activity, e.g. increased polymerase activity or a change in DNA binding by proteins that regulate transcription of the genome. Many embodiments of the invention described herein can result in random integration, especially in actively transcribed regions.

5 *Integration into Active Portions of the Genome*

Integration, however, can be directed to regions of the genome active during specific types of genome activity. For instance, integration at sites in the genome that are active during specific phases of the cell cycle can be promoted by synchronizing the cells in a desired phase of the cell cycle. Such cell cycle methods include those known in the art, such as serum deprivation or alpha factors (for yeast). Integration may also be directed to regions of the genome active during cell regulation by a chemical, such as an antagonist or agonist for a receptor or some other chemical that increase or decreases or otherwise modulates genome activity. By adding the chemical of interest, genome activity can be increased, often in specific regions to promote integration of an integration vector (e.g. as a reporter gene construct), including those of the invention, into such regions of the genome.

For instance, a nuclear receptor activator (general or specific) could be applied to activate the cells prior or during integration in order to promote integration of reporter genes at sites in the genome that become more active during nuclear receptor activation. Such cells could then be screened with the same or different nuclear receptor activator to identify which clones, and which portions of the genome are active during nuclear receptor activation. Any agonists, antagonists and modulators of the receptors described herein can be used in such a manner, as well as any other chemicals that increase or decrease genome activity.

25 *Cells for Integration into the Genome*

The cells used in the invention will typically correspond to the genome of interest. For example, if regions of the human genome are desired to be identified, then human cells containing a proper genetic complement will generally be used. Libraries, however, could be biased by using cells that contain extra-copies of certain chromosomes or other

portions of the genome. Cells that do not correspond to the genome of interest can also be used if the genome of interest or significant portions of the genome of interest can be replicated in the cells, such as making a human-mouse hybrid.

Additionally, by the appropriate choice of cells and expressed proteins,
5 identification and screening assays can be constructed that detect active portions of the genome associated with a biological process that requires, in whole or part, the presence of a particular protein (protein of interest). Cells can be selected depending on the type of proteins that are expressed (homologously or heterologously) or from the type of tissue from which the cell line or explant was originally generated. If the identification of
10 portions of the genome activated by a particular type of protein is desired, then the cell used should express that protein.

The cells can express the protein homologously, i.e. expression of the desired protein normally or naturally occurs in the cells. Alternatively, the cells can be directed to express a protein heterologously, i.e. expression of the desired protein which does not normally or naturally occur in the cells. Such heterologous expression can be directed by "turning on" the gene in the cell encoding the desired protein or by transfecting the cell with a polynucleotide encoding the desired protein (either by constitutive expression or inducible expression). Inducible expression is preferred if it is thought that the expressed protein of interest may be toxic to the cells.
15

Many cells can be used with the invention. Such cells include, but are not limited to adult, fetal, or embryonic cells. These cells can be derived from the mesoderm, ectoderm, or endoderm and can be stem cells, such as embryonic or adult stem cells, or adult precursor cells. The cells can be of any lineage, such as vascular, neural, cardiac, fibroblasts, lymphocytes, hepatocytes, cardiac, hematopoietic,
20 pancreatic, epidermal, myoblasts, or myocytes. Other cells include baby hamster kidney (BHK) cells (ATCC No. CCL10), mouse L cells (ATCC No. CCL1.3), Jurkats (ATCC No. TIB 152) and 153 DG44 cells (see, Chasin (1986) Cell. Molec. Genet. 12: 555)
25 human embryonic kidney (HEK) cells (ATCC No. CRL1573), Chinese hamster ovary (CHO) cells (ATCC Nos. CRL9618, CCL61, CRL9096), PC12 cells (ATCC No.

CRL17.21) and COS-7 cells (ATCC No. CRL1651). Preferred cells include Jurkat cells, CHO cells, neuroblastoma cells, P19 cells, F11 cells, NT-2 cells, and HEK 293 cells, such as those described in U.S. Patent No. 5,024,939 and by Stillman et al. Mol. Cell. Biol. 5: 2051-2060 (1985). Preferred cells for heterologous protein expression are those that can be readily and efficiently transfected.

- Cells used in the present invention can be from continuous cell lines or primary cell lines obtained from, for example, mammalian tissues, organs, or fluids. Tissue sections as well as disperse cells can be used in the present invention. Cells can also be obtained from transgenic animals that have been engineered to express a reporter gene.
- 10 Cells obtained from transgenic or non-transgenic animals are preferred for cells that are difficult to culture *in vitro*, such as neural and hepatic cells. Primary cell lines can be made continuous using known methods, such as fusing primary cells with a continuous cell line or expressing transforming proteins. Cells of the invention can be stored or used with methods of the invention as isolated, clonal populations in plates such as those
- 15 described in commonly owned United States Patent Applications having Attorney Docket Nos: 08366/010001, entitled "Low background multi-well plates and platforms for spectroscopic measurements" (Coassin et al., filed June 2, 1997); and 08366/009001, entitled "Low background multi-well plates with greater than 864 wells for spectroscopic measurements" (Coassin et al., filed June 2, 1997); each of which is incorporated herein
- 20 by reference. Preferably, cells are stored or used in plates with 96, 384, 1536 or 3456 wells per plate. A single cell or a plurality of cells can be placed in such wells. Such isolated clonal populations will typically have 1,000, 10,000, or 100,000 or more such populations representative of substantially equivalent numbers of independent integration sites. Such panels can be used in profiling, pathway identification,
- 25 modulator identification, modulator characterization, and other methods of the invention.

Prior to being transfected with a trapping vector of the present invention, cells can be transfected with an exogenous gene capable of expressing an exogenous protein, such as a receptor (e.g., GPCR) or gene associated with the pathology of an etiological agent, such as a virus, bacteria, or parasite. Cells that express such exogenous proteins can then

be transfected with a trapping vector to form a library of clones that can be screened using the present invention. The invention can also include animals with β -lactamase expression constructs integrated into the genome of interest.

Many of the cells of the present invention can report modulation of biological processes by a variety of additional reporter genes or chemicals or combinations thereof. For example, beta-lactamase, an enzyme, can convert non-chromogenic substrates to chromogenic products or alter the chromogenic or fluorescent properties of a substrate such as CCF2. Furthermore, fluorescent reporters, such as fluorescent proteins, such as green fluorescent protein (GFP) molecules, can be used as reporters. Some mutant GFP molecules have different fluorescent properties as compared to wild-type GFP. These GFPs can be used as reporters and can be used singly or in combination with the present invention. For example, cells can have multiple reporters that can be differentiated to report different biological processes, or different steps within a biological process, such as steps in a signal transduction pathway.

15 Targets

Proteins of interest that can be expressed in the cells of the invention include,: hormone receptors (e.g. mineralcorticosteroid, gluococorticoid, and thyroid hormone receptors); intracellular receptors (e.g., orphans, retinoids, vitamin D3 and vitamin A receptors); signaling molecules (e.g., kinases, transcription factors, or molecules such 20 signal transducers and activators of transcription) (*Science* Vol. 264, 1994, p.1415-1421; *Mol. Cell Biol.*, Vol. 16, 1996, p.369-375); receptors of the cytokine superfamily (e.g. erythropoietin, growth hormone, interferons, and interleukins (other than IL-8) and colony-stimulating factors); G-protein coupled receptors, see US patent 5,436,128 (e.g., for hormones, calcitonin, epinephrine, gastrin, and pancreatic or autocrine mediators, such 25 as stomatostatin or prostaglandins) and neurotransmitter receptors (norepinephrine, dopamine, serotonin or acetylcholine); tyrosine kinase receptors (such as insulin growth factor, nerve growth factor (US patent 5,436,128)). Examples of the use of such proteins is further described herein.

Any target, such as an intracellular or extracellular receptor involved in a signal transduction pathway, such as the leptin or GPCR pathways, can be used with the present invention. Furthermore, the genes activated or repressed by a target can be isolated, identified, and modulators of that gene identified using the present invention. For example, the present invention can identify a G-protein coupled receptor (GPCR) pathway, determine its function, isolated the genes modulated by the GPCR, and identify modulators of such GPCR modulated proteins.

As an introduction to GPCR cell biology, the activation of $G\alpha_{15}$ or $G\alpha_{16}$ can, through a G-protein signaling pathway, activate PLC β , which in turn increases intracellular calcium levels. An increase in calcium levels can lead to modulation of a "calcium-responsive" promoter that is part of a signal transduction detection system, i.e., a promoter that is activated (e.g., a NFAT promoter AP-1) or inhibited by a change in calcium levels. One example of an NFAT DNA binding site is described in Shaw, et al. Science 291:202-205 (1988). Likewise, a promoter that is responsive to changes in protein kinase C levels (e.g., a "protein kinase C-responsive promoter") can be modulated by an active $G\alpha$ protein through G-protein signaling pathway. Selected cells described herein can also include a G-protein coupled receptor. Genes encoding numerous GPCRs have been cloned (Simon et al., Science 252:802-808 (1991)), and conventional molecular biology techniques can be used to express a GPCR on the surface of a cell of the invention. Preferably, the sum responsive promoter can allow for only a relatively short lag (e.g., less than 90 minutes) between engagement of the GPCR and transcriptional activation. A preferred responsive promoter includes the nuclear factor of activated T-cell promoter (Flanagan et al., Nature 352:803-807 (1991)). Polynucleotides identified by methos of the invention can be used as response elements that are sensitive to intracellular signals (signal-response elements). Signal response elements can be used in the assays described herein, such as identification of useful chemicals. Such signal response elements may sensitive intracellular signals that include voltage, pH, and intracellular levels of Ca^{++} , ATP, ADP, cAMP, GTD, GDP, K^+ , Na^+ , Zn^{++} , oxygen, metabolites and IP3.

In one aspect of the present invention, cells can be transformed to express an exogenous receptor, such as GPCR. Such a transduced cell line can than be futher transduced with a trapping vector to make a library of clones that can be used to identify cells that report modulation of the exogenous receptor. Preferably, the host cell line 5 would not appreciably express the exogenous receptor.

Based on the unique structure of GPCRs, which have seven hydrophobic, presumably trans-membrane, domains (see, Watson and Arkinstall, The G-Protein Linked Receptor Facts Book, Academic Press, New York (1994)) orphan GPCRs (GPCRs having no known function) can be identified by searching sequence databases, such as 10 those provided by the National Library of Medicine (Bethesda, MD), for similar motifs and homologies. This same strategy can, of course, be used for any target, especially when a paradigm sequence or motif has been determined.

Drug Discovery for Viruses and Other Pathogens

The function of genes from viruses or other pathogens that effect the expression 15 of genes in cells, such as mammalian cells, can be determined using the present invention. Furthermore, chemicals that modulate these genes can be identified using the methods of the present invention. For example, many transforming viruses, after infecting a cell, have the effect of up-regulating genes involved in cell proliferation, which allows the virus-infected cells to produce additional viruses, which can infect additional cells.

20 These transforming viruses can act by stimulating a receptor from the target cell. One example of the mechanism is the Friend Erythroleukemia virus. This virus uses the erythropoietin receptor for entry into the cells. When the virus is bound to the receptor, a pathway is activated that causes an over-proliferation of red blood cells. If the activation of the erythropoietin receptor is inhibited, a decrease in the accumulation of red blood 25 cells would result which can prevent or reduce the severity of the leukemia. The development of an assay that reports the activation of mammalian target genes allows the identification of modulators of other viral or pathogenic dependent pathways. These modulators can be used as therapeutic agents.

A general procedure for establishing this assay uses the virus or an isolated viral protein as the stimulus for modulating a pathway. First, a gene-trapping library is made using a cell line that can be infected by the virus or activated by the viral protein. The virus is added to these cells, and clones are isolated that responded specifically to the 5 viral infection by the expression of a reporter gene.

As an example, the GP-120 portion of HIV protein is known to have mitogenic effect on cells exposed to GP-120, which indicates that downstream signaling pathways are being activated that can be associated with the cytotoxicity of the virus and allow its proliferation. Cell clones can be isolated that are induced by this activation which can be 10 used to screen for modulators of this cytotoxic or proliferative effect. Other viral proteins, such as NEF from HIV, can be used. Chemicals that inhibit this effect can have useful therapeutic value to treat viral infection or toxicity.

This approach can be applied to any cellular pathogen that has an effect on a target cells, such as cytotoxicity, cell proliferation, inflammation or other responses. 15 Other etiological targets include other viruses, such as retroviruses, adenovirus, papillomavirus, herpesviruses, cytomegalovirus, adeno associated viruses, hepatitis viruses, and any other virus. In addition to viruses, any other pathogen, such as parasites, bacteria, and viroids, can be used in the present invention. Particular viral targets include, but are not limited to, NEF, Hepatitis X protein, and other viral proteins, such as those 20 that can be encoded or carried by a virus. In addition, two or more viral components can be added to identify coviral pathogenesis components. This is a particularly valuable tool for identifying pathways modulated by two or more viruses concurrently, or over time as in slow activating viral conditions. For example, cotransfection with HIV and CMV may be used. Viral targets or components do not include oncogenes or proto-oncogenes found 25 in uninfected genomes, and gene products thereof.

Screening Test Chemicals Using Portions Of The Genome

Cells comprising β -lactamase polynucleotides integrated in the genome can be contacted with test chemicals or modulators of a biological process and screened for activity. Usually, the test chemical being screened will have at least one defined target,

usually a protein. The test chemical is normally applied to the cells to achieve a final predetermined concentration in the medium bathing the cells. Typically, screens are conducted at concentrations 100 uM or less, preferably 10 uM or less and preferably 1 uM or less for confirmatory screens. As described more fully herein, cells can be 5 subjected to multiple rounds of screening and selection using the same chemical in each round to insure the identification of clones with the desired response to a chemical or with different chemicals to characterize which chemicals produce a response (either an increase or decrease β -lactamase activity) in the cells. Such methods can be applied to any chemical that alters the function of any the proteins mentioned herein or known in the 10 art.

Chemicals and physiological processes without a defined target, however, can also be used and screened with the cells of the invention. For example, once a clone is identified as containing an active genomic polynucleotide that is activated by a particular cellular signal (including extracellular signals), for instance by a neurotransmitter, that 15 same clone can be screened with chemicals lacking a defined target to determine if activation by the neurotransmitter is blocked or enhanced by the chemical. This is a particularly useful method for finding therapeutic targets downstream of receptor activation (in this case a neurotransmitter). Such methods can be applied to any chemical that alters the function of any the proteins mentioned herein or known in the art. This 20 type of "targetless" assay is particular useful as a screening tool for the medial conditions and pathways described herein.

The methods and compositions described herein offer a number of advantages over the prior art. For instance, screening of mammalian based gene integration libraries is limited by the use of existing reporter systems. Many enzymatic reporter genes, such 25 as secreted-alkaline phosphatase, and luciferase, cannot be used to assay single living cells (including FACS) because the assay requires cell lysis to determine reporter gene activity. Alternatively, β -galactosidase can detect expression in single cells but substrate loading requires permeabilization of cells, which can cause deleterious effects on normal cell functions. Additionally, the properties of fluorescent β -galactosidase substrates, such

as fluorescein di- β -D-galactopyranoside, and products make it very difficult to screen large libraries for both expressing and non-expressing cells because the substrate and product is not well retained or permits ratiometric analysis to determine the amount uncleaved substrate. Green fluorescent protein (GFP), a non-enzymatic reporter, could be
5 used to detect expression in single living cells but has limited sensitivity. GFP expression level would have to be at least 100,000 molecules per cell to be detectable in a screening format and small changes in, or low levels of, gene expression could not be measured. Furthermore GFP is relatively stable and would not be suitable for measuring down-regulation of genes. Other advantages of the invention are described herein or
10 readily recognized by one skilled in the art upon reviewing this disclosure.

Methods for Rapidly Identifying Modulators of Genomic Polynucleotides

The invention provides for a method of identifying proteins or chemicals that directly or indirectly modulate a genomic polynucleotide. Generally, the method
15 comprises inserting a β -lactamase expression construct into an eukaryotic genome, usually non-yeast, contained in at least one living cell, contacting the cell with a predetermined concentration of a modulator, and detecting β -lactamase activity in the cell. Preferably, cleavage of a membrane permeant β -lactamase substrate is measured and the membrane permeant β -lactamase substrate is transformed in the cell into a
20 trapped substrate. Preferably, the β -lactamase expression construct comprises a β -lactamase polynucleotide, a splice donor, a splice acceptor and an IRES element. The method can also include determining the coding nucleic acid sequence of a polynucleotide operably linked to the β -lactamase expression construct using techniques known in the art, such as RACE.

25 Modulator Identification

Modulators described herein can be used in this system to test for an increase or decrease in β -lactamase activity in successfully integrated clones. Such cells can optionally include specific proteins of interest as discussed herein. For example, the cell can include a protein or receptor that is known to bind the modulator (e.g., a nuclear

receptor or receptor having a transmembrane domain heterologously or homologously expressed by the cell). A second modulator can be added either simultaneously or sequentially to the cell or cells and β -lactamase activity can be measured before, during or after such additions. Cells can be separated on the basis of their response to the 5 modulator (e.g. responsive or non-responsive) and can be characterized with a number of different modulators to create a profile of cell activation or inhibition.

β -lactamase activity will often be measured in relation to a reference sample, often a control. For example, β -lactamase activity is measured in the presence of the 10 modulator and compared to the β -lactamase activity in the absence of the modulator or possibly a second modulator. Alternatively, β -lactamase activity is measured from a cell expressing a protein of interest and to a cell not expressing the protein of interest (usually 15 the same cell type). For instance, a modulator may be known to bind to a receptor expressed by the cell and the β -lactamase activity in the cell is increased in the presence of the modulator compared to the β -lactamase activity detected from a corresponding cell in the presence of the modulator, wherein the corresponding cell does not express the 20 receptor.

Pathway Identification and Modulators

When a reporter gene of the invention integrates into the genome of a host cell such that the reporter gene is expressed under a variety of circumstances, these clones can 20 be used for drug discovery and functional genomics. These clones report the modulation of the reporter gene in response to a variety of stimuli, such as hormones and other physiological signals. These stimuli can be involved in a variety of known or unknown pathways that are modulated by known or unknown modulators or targets. Thus, these 25 clones can be used as a tool to discover chemicals that modulate a particular pathway or to determine a cellular pathway.

These pathways are quite varied, and fall into general classes, which have specific species, which can be modulated by known or unknown modulators or agonists or antagonists thereof. By way of example, Table 1 illustrates various pathways, species of these classes, and known modulators of these species. The invention can be used to

identify regions of the genome that are modulated by such pathways, or physiological event

5

TABLE 1
Pathways and modulators

Pathway/Physiological Event		Known Modulator
Genus	Species	
Nuclear receptors	Estrogen receptor	Estrogen
Cytokines	IL-2 receptor	IL-2
GPCRs	Vasopressin receptor	Vasopressin
Transcription factors	Fos or Jun	NFAT
Kinase dependent	Protein kinase C	PMA
Phosphatase dependent	Calcineurine	Cyclosporin A
Protease dependent	Metalloprotease	TIMPs
Chemokine	CCR1	RANTES
Ion channels	Calcium channels	Many known blockers
Second messenger dependent	Cyclic AMP	CAMP inhibitor protein
Cell differentiation	Hematopoetic development	EPO
Cell growth	IL-2 receptor	IL-2
Cell cycle dependent	CDK	P21
Apoptosis	Fas	PS3

In one embodiment, the invention provides for a genomic assay system to identify downstream transcriptional targets for signaling pathways. This method requires the target of interest to activate gene expression upon addition of chemical or expression of the target protein. A cell line that is the most similar to the tissue type where the target functions is preferred for generating a library of clones with different integration sites with β -lactamase polynucleotides or other reporter genes. This cell line may be known to elicit a cellular response, such as differentiation upon addition of a particular modulator. If this type of cell line is available, it is preferred for screening, as it represents the native context of the target. If a cell line is not available that homologously expresses the target; a cell line can be generated by heterologously expressing the target in the most relevant cell line. For instance, if the target is normally expressed in the lymphoid cells, then a

lymphoid cell line would be used generate the library.

The library of clones, as described further herein, can be separated into two pools by FACS using the FRET system described herein: an expressing pool (e.g. blue cells) and a non-expressing pool (e.g. green cells). These two pools can then be treated with a modulator followed by FACS to isolate induced clones (e.g. green to blue) or repressed clones (e.g. blue to green). Additional rounds of stimulation followed by FACS can be performed to verify initial results. The specificity of activation can be tested by adding additional chemicals that would not activate the defined target. This would allow the identification of clones that have β -lactamase polynucleotides integrated into genes activated by a variety of cellular signals.

Once a pool of cells with the desired characteristics are isolated they can be expanded and their corresponding genes cloned and characterized. Targets which could be used in this assay system include receptors, kinases, protein/protein interactions or transcription factors and other proteins of interest discussed herein.

In another embodiment, the invention provides for a method of identifying developmentally or tissue specific expressed genes. β -lactamase polynucleotide can be inserted, usually randomly, into any precursor cell such as an embryonic or hematopoietic stem cell to create a library of clones. Constitutively expressing clones can be collected by sorting for blue cells and non-expressing cells collected by sorting for green cells using the FRET system described herein. The library of clones can then be stimulated or allowed to differentiate, and induced or repressed clones isolated. Cell surface markers in conjunction with fluorescent tagged antibodies or other detector molecules could be used to monitor the expression of reference genes simultaneously. Additionally, by stimulation and sorting stem cells at various developmental stages, it is possible rapidly identify genes responsible for maturation and differentiation of particular tissues.

Additionally, clones that have a β -lactamase polynucleotide integrated, either randomly or by homologous recombination, into developmentally expressed genes can be used with FACS to isolate specific cell populations for further study, such as screening. Such methods can be used for identifying cell populations that have stem cells properties,

as well as providing an intracellular reporter that allows isolation and screening of such a population of cells.

The present invention can yield screening cell lines for a variety of targets whose downstream signaling elements are already known or postulated. These screening cell 5 lines can be used to either screen for modulators of transfected targets or as readouts for expression cloning or functional analysis of uncharacterized targets. Screening cell lines can be made for any pathway or any modulator, such those described in Table 1.

In the case of ion channels, cell lines are generated in which β -lactamase expression is used to detect a voltage change. This is possible because intracellular 10 signaling is sensitive to membrane potential and will modulate the expression of a subset of genes. In one example, a library of neuronal cells prepared following the general methods set forth in Examples 1 to 13, such as a dorsal root neuroblastoma cells, be screened for a response to a depolarization by incubating cells in high potassium (high K⁺) medium. Depending on the particular characteristics of the cell library and the 15 method used, clones with a transcriptional response to a depolarizing treatment are identified by sorting for cells which changed from either green to blue or blue to green after depolarization. These clones are designated as voltage-sensitive clones and can be used as screening cell lines to identify chemicals that modulate ion channels (either endogenously expressed or transfected) which cause a voltage change upon either activation or inhibition (e.g. K⁺ or Na⁺ channels). These cells are also useful for 20 expression cloning of ion channels. For example, a voltage-sensitive clone could be transfected with a cDNA library. Those cells transfected with functional channels that shift the membrane potential are detected via beta-lactamase and the cDNA gene products are analyzed for activity as ion channels.

Furthermore, a gene encoding a known ion channel can be transfected into the 25 voltage sensitive cell line and then used as a screen for channel modulators. For example, expression or pharmacological activation of a Na⁺ channel can cause a depolarization that can be reported by the cell line. This cell line can be used to screen for agonists or antagonists, depending on the experimental protocol of ion channel modulators. In a

variation of this approach, a genomic library from a cell line lacking K⁺ channels, such as L929 cells, can be directly transfected with a K⁺ channel gene. The expression of the K⁺ channel causes a voltage shift, such as a hyperpolarization, causing a change in expression of certain voltage-sensitive genes. The clones expressing these genes can be used to screen for regulators of the ion channel.

Orphan protein signaling pathway identification and orphan protein modulators

In another embodiment, the invention provides for a method of identifying modulators of orphan proteins or genomic polynucleotides that are directly or indirectly modulated by an orphan protein. Human disease genes are often identified and found to show little or no sequence homology to functionally characterized genes. Such genes are often of unknown function and thus encode for an "orphan protein." Usually such orphan proteins share less than 25% amino acid sequence homology with other known proteins or are not considered part of a gene family. With such molecules there is usually no therapeutic starting point. By using libraries of the herein described clones, one can extract functional information about these novel genes:

Orphan proteins can be expressed, preferably overexpressed, in living mammalian cells. By inducing over expression of the orphan gene and monitoring the effect on specific clones one may identify genes that are transcriptionally regulated by the orphan protein. By identifying genes whose expression is influenced by the novel disease gene or other orphan protein one may predict the physiological bases of the disease or function of the orphan molecule. Insights gained using this method can lead to identification of a valid therapeutic target for disease intervention.

Modulator Identification using Genomic Polynucleotides Activated by Cellular Signals

In another embodiment, the invention provides for a method of screening a defined target or modulator using genomic polynucleotides identified with the methods described herein. The gene identification methods described herein can also be used in conjunction with a screening system for any target that functions (either naturally or artificially) through transcriptional regulation.

In many instances a receptor and its ligand are known but not the downstream

biological processes required for signaling. For example, a cytokine receptor and cytokine may be known but the downstream signaling mechanism is not. A library of clones generated from a cell line that expresses the cytokine receptor can be screened to identify clones showing changes in gene expression when stimulated by the cytokine.

- 5 The induced genes could be characterized to describe the signaling pathway. Using the methods of the invention, gene characterization is not required for screen development, as identification of a cell clone that specifically responds to the cytokine constitutes a usable secondary screen. Therefore, clones that show activation or deactivation upon the addition of the cytokine can be expanded and used to screen for agonists or antagonists of
10 cytokine receptor. The advantage of this type of screening is that it does not require an initial understanding of the signaling pathway and is therefore uniquely capable of identifying leads for novel pathways.

In another embodiment, the invention provides for a method of functionally characterizing a target using a panel of clones having active genomic polynucleotides as
15 identified herein. As large numbers of specifically responding cell lines containing active genomic polynucleotides identified with a particular biological process or modulator are generated, panels containing specific clones can be used for functional analysis of other potential cellular modulators. These panels of responding cell lines can be used to rapidly profile potential transcriptional regulators. Such panels, as well as containing
20 clones with identified active genomic polynucleotides, which were generated by the invention panels, can include clones generated by more traditional methods. Clones can be generated that contain both the identified active genomic polynucleotide with a β -lactamase polynucleotide and specific response elements, such as SRE, CRE, NFAT, TRE, IRE, or reporters under the control of specific promoters. These panels would
25 therefore allow the rapid analysis of potential effectors and their mechanisms of cellular activation. A second reporter (e.g. β -galactosidase gene can also be used with this method, as well as the other method described herein.

In another embodiment, the invention provides for a method of test chemical profiling using a clone or panel of clones having identified active polynucleotides. Test

chemical characterization is similar to target characterization except that the cellular target(s) do not have to be known. This method will therefore allow the analysis of test chemical (e.g. lead drugs) effects on cellular function by defining genes effected by the drug or drug lead.

Such a method can find application in the area of drug discovery and secondary affects (e.g. cytotoxic affect) of drugs. The potential drug would be added to a library of genomic clones and clones which either were induced or repressed would be isolated, or identified. This method is analogous to target characterization except that the secondary drug target is unknown. As well as providing a screen for the secondary effects, the assay provides information on the mechanism of toxicity.

Methods Related to FACS and Identifying Active Genomic Polynucleotides

The invention provides for a method of identifying active genomic polynucleotides using clones having integrated β -lactamase polynucleotides and FACS. β -lactamase integration libraries can be used in a high-throughput screening format, such as FACS, to detect transcriptional regulation. The compatibility of β -lactamase assays with FACS enables a systematic method for defining patterns of transcriptional regulation mediated by a range of factors. This approach has not been feasible or practical using existing reporter systems. This new method will allow rapid identification of genes responding to a variety of signals, including tissue specific expression and during pattern formation.

For example, after integration of a β -lactamase polynucleotide, expressing and non-expressing cells can be separated by FACS. These two cell populations can be treated with potential modulators and changes in gene expression can be monitored using ratio-metric fluorescent readout. Pools of clones will be isolated that show either up- or down-regulation of reporter gene expression. Target genes from responding clones can then be identified. In addition, by being able to separate expressing and non-expressing cells at different time points after modulator addition, genes which are differentially regulated over time can be identified. This approach therefore enables the elucidation of transcription cascades mediated by cellular signaling. Specifically, it will provide a

means to identify downstream genes which are transcriptionally regulated by a variety of molecules including, nuclear receptors, cytokine receptors or transcription factors.

Applications of this technology are nearly unlimited in the areas of gene discovery and functional analysis. Libraries of cell lines from various tissue types could be generated and used to identify genes with specific expression patterns or regulation mechanisms. These libraries of clones would represent millions of integration sites saturating the genome and can permit the identification of any expressed gene based on its transcriptional regulation. The features of the β -lactamase reporter system, in part, allow its use for this genomic integration assay in a high-throughput format

There are a variety of other approaches that may be used with the invention, including approaches similar to those proposed for β -lactamase. Examples would include antibody epitopes presented on the cell surface with fluorescent antibodies to detect positive cells. Gel matrixes could also be used which retain secreted reporters and allow detection of positive cells. These approaches would, however, be limited in sensitivity and would not be ratiometric in their detection. They would therefore allow for only the sorting of positive cells based on fluorescent intensity.

Once active genomic polynucleotides have been identified, they can be sequenced using various methods, including RACE (rapid amplification of cDNA ends). RACE is a procedure for the identification of unknown mRNA sequences that flank known mRNA sequences. Both 5' and 3' ends can be identified depending on the RACE conditions. 5' RACE is done by first preparing RNA from a cell line or tissue of interest. This total or polyA RNA is then used as a template for a reverse transcription reactions which can either be random primed or primed with a gene-specific primer. A poly nucleotide linker of known sequence is then attached to the 3' end of the newly transcribed cDNA by terminal transferase or RNA ligase. This cDNA is then used as the template for PCR using one primer within the reporter gene and the other primer corresponding to sequence which had been linked to the 3' end of the first stand cDNA. The present invention is particularly well suited for such techniques and does not require construction of additional clones or constructs once the genomic polynucleotide has been identified.

Substrates for Measuring β -lactamase Activity

Any membrane permanent β -lactamase substrate capable of being measured inside the cell after cleavage can be used in the methods and compositions of the invention. Membrane permanent β -lactamase substrates will not require permeabilizing 5 eukaryotic cells either by hypotonic shock or by electroporation. Generally, such non-specific pore forming methods are not desirable to use in eukaryotic cells because such methods injure the cells, thereby decreasing viability and introducing additional variables into the screening assay (such as loss of ionic and biological contents of the shocked or porated cells). Such methods can be used in cells with cell walls or membranes that 10 significantly prevent or retard the diffusion of such substrates. Preferably, the membrane permeant β -lactamase substrates are transformed in the cell into a β -lactamase substrate of reduced membrane permeability (usually at least five less permeable) or that is membrane impermeant. Transformation inside the cell can occur via intracellular enzymes (e.g. esterases) or intracellular metabolites or organic molecules (e.g. sulphydryl 15 groups). Preferably, such substrates are fluorescent. Fluorescent substrates include those capable of changes, either individually or in combination, of total fluorescence, excitation or emission spectra or FRET.

Preferably, FRET type substrates are employed with the methods and compositions of the invention. Including fluorogenic substrates of the general formula I:

20 D-S-A

wherein D is a FRET donor and A is a FRET acceptor and S is a substrate for a protein with β -lactamase activity. β -lactamase activity cleaves either D-S or S-A bonds thereby releasing either D or A, respectively from S. Such cleavage resulting from β -lactamase activity dramatically increases the distance between D and A which usually causes a 25 complete loss in energy transfer between D and A. Generally, molecules of D-S-A structure are constructed to maximize the energy transfer between D and A. Preferably, the distance between D and A is generally equal to or less than the R_o .

As would readily be appreciated by those skilled in the art, the efficiency of fluorescence resonance energy transfer depends on the fluorescence quantum yield of the

donor fluorophore, the donor-acceptor distance and the overlap integral of donor fluorescence emission and acceptor absorption. The energy transfer is most efficient when a donor fluorophore with high fluorescence quantum yield (preferably, one approaching 100%) is paired with an acceptor with a large extinction coefficient at wavelengths coinciding with the emission of the donor. The dependence of fluorescence energy transfer on the above parameters has been reported Forster, T. (1948) *Ann. Physik* 2: 55-75; Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, New York: Plenum Press (1983); Herman, B., Resonance energy transfer microscopy, in: *Fluorescence Microscopy of Living Cells in Culture, Part B. Methods in Cell Biology*, Vol. 30, ed. Taylor, D.L. & Wang, Y.L., San Diego: Academic Press (1989), pp. 219-243; Turro, N. J., *Modern Molecular Photochemistry*, Menlo Park: Benjamin/Cummings Publishing Co., Inc. (1978), pp. 296-361, and tables of spectral overlap integrals are readily available to those working in the field for example, Berlman, I.B. *Energy transfer parameters of aromatic compounds*, Academic Press, New York and London (1973). The distance between donor fluorophore and acceptor dye at which fluorescence resonance energy transfer (FRET) occurs with 50% efficiency is termed R_0 and can be calculated from the spectral overlap integrals. For the donor-acceptor pair fluorescein - tetramethyl rhodamine which is frequently used for distance measurement in proteins, this distance R_0 is around 50-70 Å dos Remedios, C.G. et al. (1987) *J. Muscle Research and Cell Motility* 8:97-117. The distance at which the energy transfer in this pair exceeds 90% is about 45 Å. When attached to the cephalosporin backbone the distances between donors and acceptors are in the range of 10 Å to 20 Å, depending on the linkers used and the size of the chromophores. For a distance of 20 Å, a chromophore pair will have to have a calculated R_0 of larger than 30 Å for 90% of the donors to transfer their energy to the acceptor, resulting in better than 90% quenching of the donor fluorescence. Cleavage of such a cephalosporin by β -lactamase relieves quenching and produces an increase in donor fluorescence efficiency in excess of tenfold. Accordingly, it is apparent that identification of appropriate donor-acceptor pairs for use as taught herein in accordance with the present invention would be essentially routine to one skilled in the art.

Reporting gene substrates described in Tsien et al., PCT Publication No. WO96/30540 published October 3, 1996 are preferred for β -lactamase.

Fluorescence Measurements

When using fluorescent substrates, it will be recognized that different types of 5 fluorescent monitoring systems can be used to practice the invention. Preferably, FACS systems are used or systems dedicated to high throughput screening e.g., 96 well or greater microtiter plates. Methods of performing assays on fluorescent materials are well known in the art and are described in, e.g., Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, New York: Plenum Press (1983); Herman, B., Resonance energy transfer 10 microscopy, in: *Fluorescence Microscopy of Living Cells in Culture, Part B, Methods in Cell Biology*, vol. 30, ed. Taylor, D.L. & Wang, Y. L., San Diego: Academic Press (1989), pp. 219-243; Turro, N. J., *Modern Molecular Photochemistry*, Menlo Park: Benjamin/Cummings Publishing Co., Inc. (1978), pp. 296-361.

Fluorescence in a sample can be measured using a fluorimeter. In general, 15 excitation radiation, from an excitation source having a first wavelength, passes through excitation optics. The excitation optics cause the excitation radiation to excite the sample. In response, fluorescent proteins in the sample emit radiation that has a wavelength that is different from the excitation wavelength. Collection optics then collect the emission from the sample. The device can include a temperature controller to 20 maintain the sample at a specific temperature while it is being scanned. According to one embodiment, a multi-axis translation stage moves a microtiter plate holding a plurality of samples in order to position different wells to be exposed. The multi-axis translation stage, temperature controller, auto-focusing feature, and electronics associated with imaging and data collection can be managed by an appropriately programmed digital 25 computer. The computer also can transform the data collected during the assay into another format for presentation.

Preferably, FRET is used as a way of monitoring β -lactamase activity inside a cell. The degree of FRET can be determined by any spectral or fluorescence lifetime characteristic of the excited construct, for example, by determining the intensity of the

fluorescent signal from the donor, the intensity of fluorescent signal from the acceptor, the ratio of the fluorescence amplitudes near the acceptor's emission maxima to the fluorescence amplitudes near the donor's emission maximum, or the excited state lifetime of the donor. For example, cleavage of the linker increases the intensity of fluorescence from the donor, decreases the intensity of fluorescence from the acceptor, decreases the ratio of fluorescence amplitudes from the acceptor to that from the donor, and increases the excited state lifetime of the donor.

Preferably, changes in the degree of FRET are determined as a function of the change in the ratio of the amount of fluorescence from the donor and acceptor moieties, a process referred to as "ratioing." Changes in the absolute amount of substrate, excitation intensity, and turbidity or other background absorbances in the sample at the excitation wavelength affect the intensities of fluorescence from both the donor and acceptor approximately in parallel. Therefore the ratio of the two emission intensities is a more robust and preferred measure of cleavage than either intensity alone.

The excitation state lifetime of the donor moiety is, likewise, independent of the absolute amount of substrate, excitation intensity, or turbidity or other background absorbances. Its measurement requires equipment with nanosecond time resolution, except in the special case of lanthanide complexes in which case microsecond to millisecond resolution is sufficient.

The ratio-metric fluorescent reporter system described herein has significant advantages over existing reporters for gene integration analysis, as it allows sensitive detection and isolation of both expressing and non-expressing single living cells. This assay system uses a non-toxic, non-polar fluorescent substrate that is easily loaded and then trapped intracellularly. Cleavage of the fluorescent substrate by β -lactamase yields a fluorescent emission shift as substrate is converted to product. Because the β -lactamase reporter readout is ratiometric it is unique among reporter gene assays in that it controls for variables such as the amount of substrate loaded into individual cells. The stable, easily detected, intracellular readout eliminates the need for establishing clonal cell lines prior to expression analysis. With the β -lactamase reporter system or other analogous

systems flow sorting can be used to isolate both expressing and non-expressing cells from pools of millions of viable cells. This positive and negative selection allows its use with gene identification methods to isolate desired clones from large clone pools containing millions of cells each containing a unique integration site.

5 *High Throughput Screening System*

The present invention can be used with systems and methods that utilize automated and integratable workstations for identifying modulators, pathways, chemicals having useful activity and other methods described herein. Such systems are described generally in the art (see, U.S. Patent Nos: 4,000,976 to Kramer et al. (issued January 4, 1977), 5,104,621 to Pfost et al. (issued April 14, 1992), 5,125,748 to Bjornson et al. (issued June 30, 1992), 5,139,744 to Kowalski (issued August 18, 1992), 5,206,568 Bjornson et al. (issued April 27, 1993), 5,350,564 to Mazza et al. (September 27, 1994), 5,589,351 to Harootunian (issued December 31, 1996), and PCT Application Nos: WO 93/20612 to Baxter Deutschland GMBH (published October 14, 1993), WO 96/05488 to 10 McNeil et al. (published February 22, 1996) and WO 93/13423 to Agong et al. (published July 8, 1993).

Typically, such a system includes: A) a storage and retrieval module comprising storage locations for storing a plurality of chemicals in solution in addressable wells, a well retriever and having programmable selection and retrieval of the addressable wells 20 and having a storage capacity for at least 10,000 the addressable wells, B) a sample distribution module comprising a liquid handler to aspirate or dispense solutions from selected the addressable wells, the chemical distribution module having programmable selection of, and aspiration from, the selected addressable wells and programmable dispensation into selected addressable wells (including dispensation into arrays of addressable wells with different densities of addressable wells per centimeter squared), C) 25 a sample transporter to transport the selected addressable wells to the sample distribution module and optionally having programmable control of transport of the selected addressable wells (including adaptive routing and parallel processing), D) a reaction module comprising either a reagent dispenser to dispense reagents into the selected

addressable wells or a fluorescent detector to detect chemical reactions in the selected addressable wells, and a data processing and integration module. The addressable wells should be made of biocompatible materials that are also compatible with the assay to be performed (see, U.S. Patent Application Attorney Docket No.: 08366/008001, "Systems and methods for rapidly identifying useful chemicals in liquid samples" (Stylli et al., filed May 16, 1997), which is incorporated herein by reference.

The storage and retrieval module, the sample distribution module, and the reaction module are integrated and programmably controlled by the data processing and integration module. The storage and retrieval module, the sample distribution module, 10 the sample transporter, the reaction module and the data processing and integration module are operably linked to facilitate rapid processing of the addressable sample wells. Typically, devices of the invention can process about 10,000 to 100,000 addressable wells, which can represent about 5,000 to 100,000 chemicals, in 24-hour period. Cells clones generated using the present invention can be individually deposited into wells of a 15 multi-well platform having any number of wells, such as 96, 864, 3456, or more. The cells in the wells can be cultured, stored, screened, and inventoried using such a system.

The present invention is also directed to chemical entities and information (e.g., modulators or chemicals or databases biological activities of chemicals or targets) generated or discovered by operation of the present invention, particularly chemicals and 20 information generated using such systems.

Pharmacology and Toxicity of Candidate Modulators

The structure of a candidate modulator identified by the invention can be determined or confirmed by methods known in the art, such as mass spectroscopy. For putative modulators stored for extended periods of time, the structure, activity, and 25 potency of the putative modulator can be confirmed.

Depending on the system used to identify a candidate modulator, the candidate modulator will have putative pharmacological activity. For example, if the candidate modulator is found to inhibit T-cell proliferation (activation) *in vitro*, then the candidate modulator would have presumptive pharmacological properties as an immunosuppressant

or anti-inflammatory (see, Suthanthiran et al., Am. J. Kidney Disease, 28:159-172 (1996)). Such nexuses are known in the art for several disease states, and more are expected to be discovered over time. Based on such nexuses, appropriate confirmatory *in vitro* and *in vivo* models of pharmacological activity, as well as toxicology, can be selected. The methods described herein can also be used to assess pharmacological selectivity and specificity, and toxicity.

..... Once identified, candidate modulators can be evaluated for toxicological effects using known methods (see, Lu, Basic Toxicology, Fundamentals, Target Organs, and Risk Assessment, Hemisphere Publishing Corp., Washington (1985); U.S. Patent Nos: 10 5,196,313 to Culbreth (issued March 23, 1993) and U.S. Patent No. 5,567,952 to Benet (issued October 22, 1996). For example, toxicology of a candidate modulator can be established by determining *in vitro* toxicity towards a cell line, such as a mammalian i.e. human, cell line. Candidate modulators can be treated with, for example, tissue extracts, such as preparations of liver, such as microsomal preparations, to determine increased or decreased toxicological properties of the chemical after being metabolized by a whole organism. The results of these types of studies are often predictive of toxicological properties of chemicals in animals, such as mammals, including humans.

Alternatively, or in addition to these *in vitro* studies, the toxicological properties of a candidate modulator in an animal model, such as mice, rats, rabbits, or monkeys, can be determined using established methods (see, Lu, supra (1985); and Creasey, Drug Disposition in Humans, The Basis of Clinical Pharmacology, Oxford University Press, Oxford (1979)). Depending on the toxicity, target organ, tissue, locus, and presumptive mechanism of the candidate modulator, the skilled artisan would not be burdened to determine appropriate doses, LD₅₀ values, routes of administration, and regimes that 25 would be appropriate to determine the toxicological properties of the candidate modulator. In addition to animal models, human clinical trials can be performed following established procedures, such as those set forth by the United States Food and Drug Administration (USFDA) or equivalents of other governments. These toxicity studies provide the basis for determining the efficacy of a candidate modulator *in vivo*.

Efficacy of Candidate Modulators

Efficacy of a candidate modulator can be established using several art recognized methods, such as *in vitro* methods, animal models, or human clinical trials (see, Creasey, *supra* (1979)). Recognized *in vitro* models exist for several diseases or conditions. For example, the ability of a chemical to extend the life-span of HIV-infected cells *in vitro* is recognized as an acceptable model to identify chemicals expected to be efficacious to treat HIV infection or AIDS (see, Daluge et al., Antimicro. Agents Chemother. 41:1082-1093 (1995)). Furthermore, the ability of cyclosporin A (CsA) to prevent proliferation of T-cells *in vitro* has been established as an acceptable model to identify chemicals expected to be efficacious as immunosuppressants (see, Suthanthiran et al., supra, (1996)). For nearly every class of therapeutic, disease, or condition, an acceptable *in vitro* or animal model is available. Such models exist, for example, for gastro-intestinal disorders, cancers, cardiology, neurobiology, and immunology. In addition, these *in vitro* methods can use tissue extracts, such as preparations of liver, such as microsomal preparations, to provide a reliable indication of the effects of metabolism on the candidate modulator. Similarly, acceptable animal models may be used to establish efficacy of chemicals to treat various diseases or conditions. For example, the rabbit knee is an accepted model for testing chemicals for efficacy in treating arthritis (see, Shaw and Lacy, J. Bone Joint Surg. (Br) 55:197-205 (1973)). Hydrocortisone, which is approved for use in humans to treat arthritis, is efficacious in this model which confirms the validity of this model (see, McDonough, Phys. Ther. 62:835-839 (1982)). When choosing an appropriate model to determine efficacy of a candidate modulator, the skilled artisan can be guided by the state of the art to choose an appropriate model, dose, and route of administration, regime, and endpoint and as such would not be unduly burdened.

In addition to animal models, human clinical trials can be used to determine the efficacy of a candidate modulator in humans. The USFDA, or equivalent governmental agencies, have established procedures for such studies.

Selectivity of Candidate Modulators

The *in vitro* and *in vivo* methods described above also establish the selectivity of a candidate modulator. It is recognized that chemicals can modulate a wide variety of biological processes or be selective. Panels of cells based on the present invention can be used to determine the specificity of the candidate modulator. Selectivity is evident, for example, in the field of chemotherapy, where the selectivity of a chemical to be toxic towards cancerous cells, but not towards non-cancerous cells, is obviously desirable. Selective modulators are preferable because they have fewer side effects in the clinical setting. The selectivity of a candidate modulator can be established *in vitro* by testing the toxicity and effect of a candidate modulator on a plurality of cell lines that exhibit a variety of cellular pathways and sensitivities. The data obtained from these *in vitro* toxicity studies can be extended animal model studies, including human clinical trials, to determine toxicity, efficacy, and selectivity of the candidate modulator.

The selectivity, specificity and toxicology, as well as the general pharmacology, of a test chemical can be often improved by generating additional test chemicals based on the structure/property relationships of the test chemical originally identified as having activity (a "Hit"). Test chemicals identified as having activity can be modified to improve various properties, such as affinity, life-time in the blood, toxicology, specificity and membrane permeability. Such refined test chemicals can be subjected to additional assays as described herein for activity analysis. Methods for generating and analyzing such chemicals are known in the art, such as U.S. patent 5,574,656 to Agrafiotis et al.

Compositions

The present invention also encompasses a modulator in a pharmaceutical composition comprising a pharmaceutically acceptable carrier prepared for storage and subsequent administration, which have a pharmaceutically effective amount of the candidate modulator in a pharmaceutically acceptable carrier or diluent. Chemicals identified by the methods described herein do not include chemicals publicly available as of the filing date of the present application or in the prior art. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R.

Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used.

5 The compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; and the like. Injectables can be prepared in conventional forms either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable 10 excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (e.g., liposomes), may be utilized.

15 The pharmaceutically effective amount of the candidate modulator required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will 20 recognize. In practicing the methods of the invention, the pharmaceutical compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in vitro*. In employing them *in vivo*, the pharmaceutical composition can be administered to the mammal in a variety of ways, 25 including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. Such methods may also be applied to testing chemical activity *in vivo*.

As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age,

weight and mammalian species treated, the particular pharmaceutical composition employed, and the specific use for which the pharmaceutical composition is employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine methods as discussed above. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable *in vitro* studies can be used to establish useful doses and routes of administration of the compositions identified by the present methods using established pharmacological methods.

In non-human animal studies, applications of potential pharmaceutical compositions are commenced at higher dosage levels, with the dosage being decreased until the desired effect is no longer achieved or adverse side effects are reduced or disappear. The dosage for the products of the present invention can range broadly depending upon the desired affects and the therapeutic indication. Typically, dosages may be between about 10 ng/kg and 1 μ g/kg body weight, preferably between about 100 μ g/kg and 10 mg/kg body weight. Administration is preferably oral on a daily basis.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (see e.g., Fingl et al., in The Pharmacological Basis of Therapeutics, 1975). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, organ dysfunction, or other adverse effects. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

.....

Depending on the specific conditions being treated, such pharmaceutical compositions may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may 5 include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

For injection, the pharmaceutical compositions of the invention may be 10 formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Use of pharmaceutically acceptable carriers to formulate the pharmaceutical compositions herein disclosed for the 15 practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The pharmaceutical compositions can be formulated readily using pharmaceutically acceptable carriers well 20 known in the art into dosages suitable for oral administration. Such carriers enable the chemicals of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using 25 techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external micro-environment and, because liposomes fuse with cell membranes, are efficiently delivered

into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amount of a pharmaceutical composition is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active chemicals into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping, or lyophilizing processes. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active chemicals in water-soluble form. Additionally, suspensions of the active chemicals may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the chemicals to allow for the preparation of highly concentrated solutions.

Pharmaceutical compositions for oral use can be obtained by combining the active chemicals with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose,

hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, 5 concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dye-stuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active chemical doses.

.....

EXAMPLES

Example 1 β -lactamase Expression Constructs

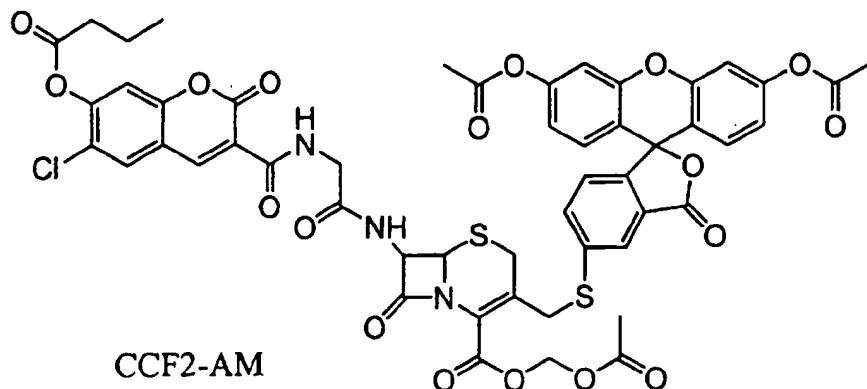
To investigate various beta-lactamase expression constructs (**BLECs**) multiple **BLECs** were constructed and transfected into mammalian cells.

5 The first of these, **BLEC-1** was constructed by cloning the cytoplasmic form of β -lactamase SEQ.ID NO. 4 (see Table 1) such that it is functionally linked to the En-2 splice acceptor sequence, as shown in FIG. 3. This vector when inserted into a genomic intron will result in the generation of a fusion RNA between an endogenous target gene and β -lactamase ("BL"). **BLEC-1** also 10 contains a bovine growth hormone poly-adenylation sequence (BGH-polyA) downstream of the cytoplasmic Beta-lactamase.

15 **BLEC-2** was constructed identically to **BLEC-1**, except that a poliovirus internal ribosomal entry site (IRES) sequence was inserted between the En-2 splice acceptor β -lactamase ("BL"). This eliminates reading frame restrictions and possible inactivation of beta-lactamase by fusion to an endogenous protein. To allow for selection of stable transfectants for **BLEC-1** and **BLEC-2** a neomycin or G418 resistance cassette was cloned downstream of the BGH poly-adenylation sequence. This cassette consists of a promoter, neomycin resistance gene and an SV40 poly-adenylation sequence, as shown in FIG. 3.

20 Two alternative constructs **BLEC-3** and **BLEC-4** were constructed similar to **BLEC-1**, and **BLEC-2** respectively, except the SV40-poly A was replaced with a splice donor sequence. This should enrich for insertion into transcribed regions, as it requires the presence of an endogenous splice acceptor and polyadenylation sequence downstream of the vector insertion site to generate 25 G418 resistant clones. **BLEC-3** and **BLEC-4** also use the PGK promoter to drive the neomycin resistance gene instead of the human beta-actin promoter.

The structure of CCF2-AM (BL substrate) used in the experiments below is:



5

Table 2

SEQ. ID NO.	parent -BL gene and reference	modification	mammalian expression vector	location of expression
# 1	<i>Escherichia coli</i> RTEM Kadonaga et al.	Signal sequence replaced by: ATG AGT	pMAM-neo glucocorticoid-inducible	cytoplasmic
# 2	<i>Escherichia coli</i> RTEM Kadonaga et al.	Wild type secreted enzyme 2 changes in pre-sequence: ser 2 arg , ala 23 gly	pMAM-neo glucocorticoid-inducible	secreted extracellularly
# 3	<i>Escherichia coli</i> RTEM	-globin up stream leader: AAGCTTTTGCAAGCTCA GAATAAACGCAACTTCCG Kozak sequence: GGTACCAACCATGG signal sequence replaced by: ATG GGG	pCDNA 3 CMV promotor and pZEO SV40 promotor	cytoplasmic
# 4	<i>Escherichia coli</i> RTEM	Kozak sequence: GGTACCAACCATGG signal sequence replaced by: ATG GAC (GAC replaces CAT)	pCDNA3 CMV promoter AND BLECs	cytoplasmic
# 5	<i>Bacillus licheniformis</i> 749/C Neugebauer et al.	signal sequence removed, new N-terminal ATG	pCDNA 3 CMV promotor	cytoplasmic

Table 3

Functional Elements

	Splice acceptor	Adapter	Reporter gene	Reporter gene poly A	Selection Promoter	Resistant Marker poly A
VECTORS						
BLEC-1	En2-splice acceptor	protein fusion	SEQ. ID NO. 4	BGH polyA	β-actin promoter	Neo polyA
BLEC-2	En2-splice acceptor	IRES	SEQ. ID NO. 4	BGH polyA	β-actin promoter	Neo-polyA
BLEC-3	En2-splice acceptor	Protein fusion	SEQ. ID NO. 4	BGH polyA	PGK promoter	Neo-splice donor
BLEC-4	En2-splice acceptor	IRES	SEQ. ID NO. 4	BGH polyA	PGK promoter	Neo-splice donor

5

Example 2 Libraries of BLEC Clones

To investigate the function of each of the **BLEC** vectors they were transfected by electroporation into RBL-1 cells and stable clones were selected for each of the four **BLEC** plasmids (see Table 2). Selective media contained 10 DMEM, 10% fetal bovine serum (FBS) and 400 µg/ml Geneticin (G418). G418 resistant cell clones were pooled from multiple transfections to generate a library of **BLEC** stable integrated clones.

This library of **BLEC-1** integrated clones was loaded with the fluorescent substrate of **BL** (CCF-2-AM) by adding 10µM CCF-2-AM in HBSS containing 15 10µM hepes 7. 1 and 1% glucose. After a 1 hour incubation at 22°C cells were washed with HBSS and viewed upon excitation with 400nm light using a 435nm long pass emission filter. Under these assay conditions 10% of the cells were blue fluorescent indicating they were expressing β-lactamase. This result suggests that that **BLEC-1** construct is functioning as a gene integration vector.

20 Stable cell lines were also generated by transfecting **BLEC-1** into **CHO-K1** and **Jurkat** cells. Populations of **BLEC-1** integrated clones from **CHO** and **Jurkat** cells showed similar results to those obtained with **RBL-1** clones with 10-15 % of **BLEC** integrated cell clones expressing **BL** as determined by their

blue/green ratio after loading with CCF-2-AM. This result shows that the BLECs function in a variety of cell types including human T-cells (Jurkat), rat basophilic leukocytes (RBL), and Chinese hamster ovarian (CHO).

5 **Example 3 Isolating BLEC Clones Expressing β -lactamase**

Fluorescent activated cell sorting of multi-clonal populations of RBL-1 gene integrated clones was used to identify clones with regulated BL gene expression. A BL non-expressing population of cells was isolated by sorting a library of BLEC-1 integrated clones generated by transfection of RBL-1 cells as described in Example 2. 180,000 clones expressing little or no BL were isolated by sorting for clones with a low blue/green ratio (R1 population), as shown in FIG. 4A. This population of clones was grown for seven days and resorted by FACS to test the population's fluorescent properties. FACS analysis of the cell clones sorted from R1 shows that most of the cells with a high blue/green ratio ~0.1% have been removed by one round of sorting for green cells, as shown in FIG. 4B. It is also clear that the total population has shifted towards more green cells compared to the parent population, as shown in FIG. 4A. There are, however, cells with a high blue/green ratio showing up in the green sorted population. These may represent clones in which the BLEC has integrated into a differentially regulated gene such as a gene whose expression changes throughout the cell cycle.

The population of RBL-1 clones shown in FIG. 4B was stimulated by addition of 1uM ionomycin for 6 hours and resorted to identify clones which had the BLEC integrated into a gene which is inducible by increasing intracellular calcium. Table 3 below summarizes the results from this experiment. A greater percentage of blue clones were present in all three of the blue sub-population (R4, R2, R5) in the ionomycin stimulated when compared to the unstimulated population. This sorted population represents the following classes of blue cells: R4 (highest blue/green ratio (bright blues)), R2 (multicolor blues), and R5 (lower

blue/green ratio (least blue). Additionally, in the ionomycin stimulated population there is a decrease in the percent green cells from the unstimulated population (R6). This increase in blue clones in the ionomycin stimulated population indicates that a sub-population of blue clones have the BLEC inserted into a gene which is induced by ionomycin. Individual blue clones were sorted from the ionomycin stimulated population and are analyzed for their expression profile.

Table 4
Sort Window (See FIG. 4)

		R4 (blue)	R2	R5	R6 (green)
10	Unstimulated %	.11	2.39	1.53	66.23
IuM Ionomycin	Stimulated %	.24	3.5	2.5	61.64
Ratio +Ion-/Ion		2.2	1.5	1.6	.9

In addition to allowing the isolation of cell clones with inducible BL expression from large populations of cells, clones can be isolated based their level of BL expression. To isolate cells with different levels of BL expressions blue clones can be sorted after different exposure times to substrate or by their blue/green ratio. Cell with a lower blue/green ratio or those requiring longer incubation times will represent clones expressing lower levels of BL. This is demonstrated by the FACS scan above as clones sorted from the R4 window have a higher blue/green ration indicating they are expressing higher levels of BL, cells sorted from the R5 have a lower blue/green ratio (visually turquoise) indicating lower BL expression. Cell sorted from the R3 window which contain all the blue cells show variation in blue color from bright blue (high blue/green ratio) to turquoise blue (low blue/green ratio).

To demonstrate that the expression constructs are relatively stable for sorted clones cells were sorted from R3 (blue population) as shown in FIG. 4A

and cultured in the absence of selective pressure for several weeks. There was little change in the percent of blue cells in the cultured population with the percent blue being maintained at ~90%. This result represents a 10-fold enrichment for clones constitutively expressing **BL** by one round of FACS selection.

5 Cells in R6 window have the lowest blue/green ration and appear green visually. R6 cell is therefore not expressing **BL** or are expressing **BL** below the detection limit of our assay.

Example 4 Stability of BLEC Clones

10 To further investigate the stability of reporter gene integrations into constitutively active genes, single blue clones were sorted from cell clone populations generated by transfecting RBL-1, and CHOK1 with **BLEC-1**. After addition of CCF-2 to the multi-clonal cell population, single blue clones were sorted into 96 well microtiter plates. These clones were expanded to 24 well dishes which took 7-10 days. The cell viability varied between the two cell types with 80% of the sorted clones forming colonies for the CHO and 36% for the RBL-1 cells. After expansion into a 24 well dishes 20 CHO **BLEC-1** stable clones were tested for **BL** expression by addition of CCF-2 -AM. 20/20 of these clones expressed **BL** with the percent blue cells within a clone ranging from 70%
15 to 99%. This result is consisted with the earlier data presented for RBL-1 in which the blue sorted population was tested for **BL** expression after several weeks of non-selective culturing. There was however a significant differences between clones in their blue/green ratio and hence their level of **BL** expression. This suggested that genes with different levels of constitutive expression had been
20 tagged with the **BLEC**. Although there was a significant differences in blue color between separate clones the blue fluorescence within a clone was consistently similar as would be expected in a clonal population. There were however green cells within the blue sorted clones, which may indicate that there is some loss of
25 the **BLEC-1** plasmid integration site when clones are grown up from a single cell.

Single clones were expanded and used to make RNA for RACE to identify the target gene and DNA for southern analysis.

5 **Example 5 Isolation of Jurkat BLEC integrated clones that constitutively express
beta-lactamase**

Jurkat cells are a T-cell line derived from a human T-cell leukemia. This cell line maintains many of the signaling capabilities of primary T-cells and can be activated using anti-CD3 antibodies or mitogenic lectins such as phytohemagglutinin (PHA). Wild type Jurkat cells were transfected by electroporation with a beta-lactamase trapping construct 10 (**BLEC-1, BLEC-1A, or BLEC-1B** see FIG. 3) ("BLEC constructs") that contains a gene encoding an beta-lactamase gene that is not under control of a promoter recognized by the Jurkat cells and a neomycin resistance gene that can be expressed in Jurkat cells. **BLEC-1** is set forth in FIG. 3. **BLEC-1A** has a NotI site after the SV40 poly A site. This allows the cutting of the insert away form the plasmid backbone. **BLEC-1B** is the 15 same as **BLEC-1A** except that the ATG at the beta-lactamase translation start has been changed to ATC. This eliminated the translation start site and requires the addition of an upstream ATG to produce beta-lactamase. Stable transformants were selected for their resistance to 800 μ g/ml G418. After 400 separate experiments, a pool of greater than one million clones with **BLEC** insertions was produced. This population of cells is a library 20 of cell clones in which the **BLEC** construct inserted throughout the genome ("Jurkat **BLEC** library"). Approximately ten percent of the cells in this library express beta-lactamase in the absence of added stimuli. Beta-lactamase activity in the cells was determined by contacting the cells with CCF2-AM and loading in the presence of Pluronic 128 (from Sigma) at a about 100 μ g/ml.. Individual clones or populations of 25 cells that express beta-lactamase can be obtained by FACS sorting.

Genomic Southern analysis of these clones using a DNA probe encoding beta-lactamase showed the vector inserted into the host genome between one and three times per cell, with most clones having one or two vector insertion sites (for Genomic Southern analyses, see Sambrook, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor

Laboratory Press (1989)). Northern analysis of these clones using a DNA probe that encodes beta-lactamase showed that the level of expression and message size varied from clone to clone (for Northern analysis, see Sambrook, *supra*, (1989)). This indicated that fusion transcripts were being made with different genes functionally tagged with beta-lactamase, which allows for the reporter gene to be expressed under the same conditions as the endogenous gene. Using appropriate primers, RACE (Gibco BRL) was used to isolate the genes linked to the expressed beta-lactamase gene in a subset of these constitutively expressing clones. These genes were cloned and sequenced using known methods (see, Sambrook, *supra*, (1989)). These sequences were compared with known sequences using established BLAST search techniques. Known sequences that were identified included: beta-catenin, moesin, and β -adaptin. Additionally, several novel sequences were identified which represent putative genes.

15 **Example 6 Isolation of Jurkat BLEC integrated clones that show induced expression of beta-lactamase upon activation**

Jurkat BLEC integrated clones that exhibit beta-lactamase expression upon activation of the Jurkat cells by PHA (PHA induced clones) were isolated by FACS sorting a Jurkat BLEC library. These clones represent cells in which the trapping construct had integrated into a gene up regulated by PHA (T-cell) activation. Thus, these 20 cells report the transcriptional activation of a gene upon cellular activation. Individual clones were identified and isolated by FACS using CCF2-AM to detect beta-lactamase activity. This clone isolation method, the induced sorting paradigm, used three sequential and independent stimulation and sorting protocols. A FACS read out for Jurkat cells that don't contain a BLEC construct contacted with CCF2-AM was used as a control. These 25 control cells were all green.

The first sorting procedure isolated a pool of blue (β -lactamase expressing, as indicated by contacting the cells with CCF2-AM) clones which had been pre-stimulated for 18 hours with 10 μ g/ml PHA from an unsorted Jurkat BLEC library. This pool represented 2.83 % of the original unsorted cell population. This selected pool contained

clones that constitutively express beta-lactamase and clones in which the beta-lactamase expression was induced by PHA stimulation ("stimulatable clones"). After sorting, this pool of clones was cultured in the absence of PHA to allow the cells, in the case of stimulatable clones, to expand and return to a resting state (i.e. lacking PHA induced gene expression).

The second sorting procedure isolated a pool of green (non- β -lactamase expressing, as indicated by contacting the cells with CCF2-AM) cell clones from the first sorted pool that had been grown, post-sorting, without PHA stimulation for 7 days. The second sorting procedure separates clones that constitutively express beta-lactamase from 10 cells that express beta-lactamase upon stimulation. This second pool represented 11.59% of the population of cells prior to the second sort. This pool of cells was cultured in the absence of PHA to amplify the cell number prior to a third sort.

The third sorting procedure used the same procedure as the first sorting procedure and was used to isolate individual cells that express beta-lactamase in response to being 15 contacted with 10 μ g/ml PHA for 18 hours. Single blue clones were sorted individually into single wells of 96 well microtiter plates. This three round FACS sorting procedure enriched PHA inducible clones about 10,030 fold.

These isolated clones were expanded and tested for PHA inducibility by microscopic inspection with and without PHA stimulation in the presence of CCF2-AM. 20 A total of fifty-five PHA inducible clones were identified using this procedure. The PHA inducibility for these clones ranged from a 1.5 to 40 fold change in the 460/530 ratio as compared to unstimulated control cells. Genomic Southern analysis using a DNA probe encoding beta-lactamase established that these clones represented 34 independent stable vector integration events. A list of clones obtained by the methods of the present 25 invention and their characteristics is provided below in **Table 6** and **Table 7**.

In addition to PHA inducible clones, Phorbol 12-myristate 13-acetate (PMA) (Calbiochem), Thapsigargin (Thaps) (Calbiochem), and PMA + Thaps inducible clones were isolated using the general procedure set forth above using the indicated inducer rather than PHA. PMA is a specific activator of PKC (protein kinase C) and Thaps is a

specific activator of intracellular calcium ion release (Thaps). These clones were isolated using three rounds of FACS using the general procedures described for the PHA inducible clones in Example 5. In such instances, other stimulants were substituted for PHA. PMA was provided at 8 nM, Thaps was provided at 1 μ M. When these two stimulants were combined, their concentration was not changed. As shown in Table 5, clones were selected based on their activation by PMA, Thaps, or PMA with Thaps after three or eighteen hours of stimulation ("stimulation time"). These results demonstrate that the FACS sorting criteria can be varied depending upon the type of modulated clones desired. By using varied selection conditions, it is possible to isolate functionally distinct clones downstream of the desired signaling target.

Example 7 Isolation of Jurkat BLEC integrated clones that show repressed expression of beta-lactamase upon activation

Jurkat BLEC clones that exhibit decreased beta-lactamase expression upon activation of the Jurkat cells by PHA were isolated by FACS sorting. These clones represent cells in which the BLEC trapping construct had integrated into a gene down regulated by PHA (T-cell) activation. Thus, these cells report the transcriptional repression of a gene upon cellular activation. Individual clones were identified and isolated by FACS using CCF2-AM to detect beta-lactamase activity using the following repressed sorting paradigm.

A first sort was used to isolate a population of cells that constitutively express beta-lactamase by identifying and isolating a population of blue cells from an unstimulated population of BLEC transfected Jurkat cells contacted with CCF2-AM. The sorted population of cells represented 2.89% of the unsorted population. These cells were cultured, divided into two pools, and stimulated with one of two different stimuli, either 10 μ g/ml PHA for 18 hours, or 8 nM PMA and 1 μ M Thapsigargin for 18 hours. These stimulated cells were contacted with CCF2 (loading in the presence of 400 PET (4% weight/volume) and Pluoronic 128 (100 μ g/ml)) and the green cells in the population were sorted using FACS. The sorted population represented 8.41 % of the cell

population prior to the second sort. The third round of FACS was for single blue unstimulated cells. The population of cells obtained represented 18.2 % of the cell population prior to the third sort.

This sorting procedure represents a 2,260-fold enrichment for PHA repressible clones. These clones have the beta-lactamase gene integrated into a gene that is down regulated by PHA stimulation of the cells. Six of 80 individual clones tested were repressed by PHA or PMA + Thapsigargin. All of these clones were confirmed to be independent integration events by genomic Southern analysis using a DNA probe encoding beta-lactamase. The results of these studies are presented in Table 5.

10

TABLE 5

Identification of trapping cell lines with reporter genes expression which is regulated by T-cell activation

Stimuli (Dose)	First Sort Activation Chemical and Time of Exposure	Stimulation Time	Sorting Paradigm	Clones Isolated	Clones with One or Two Vector Insertion(s)	
					1	2
PHA (10 µg/ml)	PHA 18 hours	18 hours	Induced	34	24	10
PMA (8 nM) + Thaps (1 µM)	PMA + Thaps 3 hours	3 hours	Induced	2	2	0
PMA (8 nM)	PMA 3 hours	3 hours	Induced	3	2	1
Thaps (1 µM)	Thaps 3 hours	3 hours	Induced	2	2	0
PHA (10 g/ml) or PMA (8nm) + Thaps (1 µM)	No Stimulation	18 hours	Repressed	6	5	1

15

Example 8 Specificity of T-cell modulated clones

Isolated clones from PHA-induced (Example 6) and PHA-repressed (Example 7) procedures described above were characterized to determine the specificity of their modulation and time required for induction or repression. Clones were stimulated with multiple activators or inhibitors over a one to twenty-four hour time interval. As shown in Table 6, five clones produced by the induced and repressed sorting paradigms using a

plurality of activators were tested for their responsiveness to a variety of T-cell activators, suppressors, and combinations thereof.

5

TABLE 6
Sorting protocols and specificity of activated BLEC Jurkat clones

Clone	Sorting Procedures				Relative Beta-Lactamase Activity of the Clone by the Indicated Stimulus After 24 hours (% of maximum activated stimuli)							
	Paradigm	First Sort Stimulus and (cell color sorted for)	Second Sort Stimulus and (cell color sorted for)	Third Sort Stimulus And (cell color sorted for)	None	PMA (8nM)	Thaps (1μM)	PMA (8nM) + Thaps (1μM)	PMA (8nM) + Thaps (1μM) + CsA (100 nM)	PHA (10 μg/ml)	PHA (10 μg/ml) + CsA (100 nM)	
J83-PT19	Induced	PHA ^a (blue)	N/S (green)	PHA (blue)	0	<1	100	50	<5	60	<5	
J32-6D4	Induced	PHA (blue)	N/S (green)	PHA (blue)	0	60	1-2	100	70	80	75	
C2	N/S	N/S	N/S	N/S	0	<1	0	100	<1	30	1	
J389-PT14	Induced	PMA ^b + Thaps ^c (blue)	N/S (green)	PMA + Thaps (blue)	0	90	5	85	100	85	90	
J83 97-PPTR2	Repressed	N/S (blue)	PMA + Thaps (green)	N/S (blue)	0	100	85	-50	85	67	75	
J83-PT18	Induced	PHA (blue)	N/S (green)	PHA (blue)	0	80	100	25	70	60	60	

"N/S" means "no stimulation"

^a concentration of PHA used was 10 μg/ml.

10 ^b concentration of PMA used was 8 nM.

^c concentration of Thaps used was 1 μM

In this study, PMA, which is a PKC activator, Thapsigargin which increases intracellular calcium, PHA which activates the T-cell receptor pathway, and cyclosporin 15 A which is a clinically approved immunosuppressant that inhibits the Ca²⁺ dependent phosphates calcineurin were investigated for their ability to modulate beta-lactamase expression in PHA induced and repressed BLEC clones.

The selected clones show varied dependence for their activation and inhibition by these activators and inhibitors which give an indication of the signaling events required 20 for their transcriptional activation. Five of the listed clones were generated using the

approaches described above in Example 6. The clone C2 was generated using a more classical approach. This clone was generated by transfecting a plasmid construct in which a 3X NFAT response element has been operably linked to beta-lactamase expression. This 3XNFAT element represents a DNA sequence that is present in the promoter region 5 of IL-2 and other T-cell activated genes. In addition the C2 cell line has been stably transfected with the M1 muscarinic receptor. This allows the activation of beta-lactamase expression in this clone using an M1-muscarinic agonist such as carbachol. This cell line therefore represents a good control for the cellular activators and inhibitors tested as the signaling events required for its activation are established.

10 The results of these studies indicate that the cell lines generated vary in their specificity towards activation or repression by activators. Thus, depending on the type of system that these cells are to be used to investigate, a panel of clones with varying specificity towards a specific pathway are made available by the present methods.

15 **Table 7 and Table 8** provide data similar to that provided in **Table 5** for all of the clones obtained by the methods of Examples 5 to 7.

TABLE 7
Characterization of induced BLEC Jurkat clones

CLONE Number	TIME (hours) for first detectable change in color	Change in 460/530 ratio in the indicated clone by the following activator				
		PHA (10 ⁻⁴ g/ml)	Thaps (1μM)	PMA (8 nM)	PMA (8 nM) + Thaps (1μM)	Anti-CD3 (2μg/ml) (Pharmingen)
J325B5	6	7	Nt	2-3	Nt	4-5
J325B11	6	9	1-2	2-3	Nt	5-6
J325E3	6	7	Nt	2-3	Nt	4-5
J325G4	6	3-4	Nt	3-4	Nt	4-5
J326E6	6	11	Nt	3-4	Nt	6
J326C9	6	4-5	1-2	2-3	Nt	3-4
J325E1	<2	8	Nt	8	Nt	5-6
J326D4	<2	10	0	10	Nt	5-6
J326D7	<2	10	Nt	10	Nt	5-6
J326F7	<2	10	Nt	10	Nt	5-6
J326H4	<2	10	Nt	10	Nt	5-6
J83P11	Nt	3-4	3-4	3-4	4-5	2-3
J83P12	5-6	8	1-2	7-8	7-8	3-4
J83P18	5-6	4-5	1-2	4-5	4-5	2-3
J83P13	5-6	5-6	6-7	3-4	5-6	2-3
J83P14	4-6	3-4	3-4	0	2-3	2

CLONE Number	TIME (hours) for first detectable change in color	Change in 460/530 ratio in the indicated clone by the following activator				
		PHA (10 µg/ml)	Thaps (1 µM)	PMA (8 nM)	PMA (8 nM) + Thaps (1 µM)	Anti-CD3 (2 µg/ml) (Pharmingen)
J83PI6	6-18	6-7	7-8	0	4-5	4
J83PI9	6	6	5-6	0	4-5	3-4
J83PI5	Nt	Nt	Nt	Nt	Nt	Nt
J83PI7	6-18	2	2	2	2	1.5-2
J83PI15	Nt	3-4	2	3-4	3-4	3-4
J83PI16	Nt	3-4	1-2	3-4	3-4	2-3
J83PI18	Nt	5-6	7-8	5	Nt	Nt
J83PI12	Nt	Nt	Nt	Nt	Nt	Nt
J83PI14	Nt	2	2	2	Nt	Nt
J83PI17	Nt	Nt	Nt	Nt	Nt	Nt
J83PI19	Nt	5-6	1-2	3	1-2	1-2
J83PI11	Nt	Nt	Nt	Nt	Nt	Nt
J83PI13	Nt	2-3	2-3	0	Nt	Nt
J97PI1	Nt	3-4	3-4	3-4	3-4	3-4
J97PI2	Nt	2-3	Nt	Nt	2-3	Nt
J97PI3	Nt	1-2	1-2	1-2	1-2	Nt
J97PI4	Nt	1-2	1-2	1-2	1-2	Nt
J97PI5	Nt	1.5	1.9	1.5	2-3	Nt
J97PI6	Nt	3-4	4-6	1-2	4-6	Nt
J97PI13	Nt	2-3	5-6	1-2	4-5	Nt
J97PI18	Nt	1-2	3-4	1-2	4-5	Nt
J97PI7	Nt	3-4	4-5	1-2	5-6	Nt
J97PI17	Nt	4-5	7-8	1-2	8-10	Nt
J97PI8	Nt	2.5-3	3-4	1-2	3-4	Nt
J97PI9	Nt	2-3	4-5	1-2	5-6	Nt
J97PI10	Nt	3-4	3-4	1-2	4-5	Nt
J97PI23	Nt	4-5	4-5	1-2	4-5	1-2
J97PI11	Nt	3-4	5-6	2	4-5	Nt
J97PI15	Nt	1-2	3-4	1-2	3-4	Nt
J97PI12	Nt	3-4	5-6	2-3	5-6	Nt
J97PI22	Nt	5-6	5-7	2-3	3-4	3-4
J97PI14	Nt	4-5	3-4	2	4-5	Nt
J97PI16	Nt	2-3	3-4	2-3	4	Nt
J97PI19	Nt	2-3	2-3	1-2	2-4	Nt
J97PI20	Nt	1-2	2-3	1-2	1-2	Nt
J97PI21	Nt	2-3	2-3	1-2	2-3	2-3
J97PI24	Nt	3-4	3-4	2-3	7-10	3-4
J389PT1	2hours	5-6	3-4	8-9	8-9	3-4
J389PT4	1hour	15	10	12	16	15
J389PM2	1hour	4-5	3-4	3-4	4-5	4-5
J389PM3	1hour	3-4	2-3	2-3	3-4	3-4
J389PM5	1hour	4-5	3-4	3-4	4-5	4-5
J389PM7	3hours	1-2	2-3	1-2	1-2	1-2
J389PM8	2-3hours	2-3	3-4	2-3	2-3	3-4
J389T11	3-5hours	1-2	2-3	1-2	2-3	2-3
J389T14	2hour	0	3-4	1-2	2-3	0

"Nt" means "not tested"

TABLE 8
Characterization of repressed BLEC Jurkat clones

CLONE #	Relative repression of beta-lactamase in the indicated clone by the following activator			
	PHA (10 μ g/ml)	PHA (10 μ g/ml) + CsA (100 nM)	PMA (8 nM) + Thaps (1 μ M)	PMA (8 nM) + Thaps (1 μ M) + CsA (100 nM)
J83/97pptr1	90	90	75	75
J83/97pptr2	10	-60	10	-80
J83/97pptr3	10	-50	10	-100
J83/97pptr4	60	60	40	70
J83/97pptr5	50	60	50	50
J83/97pptr6	70	70	70	70

5

To confirm that changes in reporter gene activity reflected changes in mRNA expression in these clones, Northern analysis was performed on induced, constitutive, and repressed clones using a radio labeled DNA probe directed towards the beta-lactamase gene. All clones that had beta-lactamase enzyme inducibility tested showed beta-lactamase mRNA inducibility. All clones that showed constitutive expression of beta-lactamase showed constitutive expression of beta-lactamase mRNA. All clones that showed repressed beta-lactamase expression showed repressed beta-lactamase mRNA. The message size of the control beta-lactamase mRNA was about 800 base pairs. The sizes of some from other beta-lactamase clones of the RNA were shifted higher in the gel, indicating a fusion RNA had been made between the endogenous transcript and beta-lactamase. Two known genes, CDK-6 (isolated from clone J83-PTI1) and Erg-3 (isolated from clone J89-PTI4), and two unknown genes were identified, which were isolated from clones J83PI15 and J83PI2, respectively. For clone J389-PTI4, a Northern blot was performed with the Erg-3 probe made using appropriate PCR primers determined from a published sequence which hybridizes with both the fusion RNA and the wild type RNA (for the sequence of Erg-3 see Stamminger et al., *Int. Immunol.* 5:63-70 (1993); for PCR methodologies, see U.S. Patent Nos: 4,800,159, 4,683,195, and

4,683,202). The inducibility in wild type Jurkat cells mimicked the beta-lactamase activity in this clone.

5 **Example 9 Screening of a library of known pharmacologically active modulators using a T-cell activated BLEC clone**

T-cell clone J32-6D4 was used to identify potential inhibitors of the T-cell receptor pathway. This clone was selected for further study because it is difficult to identify chemicals that inhibit specific T-cell receptor pathway. Thus, this clone was used to identify chemicals that inhibit this T-cell receptor pathway that is also stimulated by the PKC activator PMA.

A first screen was performed using a generic set of 480 chemicals with known properties. The chemicals in this set were known to have pharmacological activity. Approximately one percent (7/480) of these chemicals showed greater than 50% inhibition of the PHA activation of beta-lactamase expression in clone J32-6D4 when tested in duplicate at 10 μ M of chemical. Cells were activated with 1 μ g/ml of PHA for 18 hours in the presence of test chemicals to test for inhibitory activity. The seven chemicals that specifically inhibited clone J32-6D4 are shown in Table 9. Two of these chemicals specifically inhibited clone J32-6D4 and not the control C2 cell line. This assay for the specificity of inhibition included screening these 480 chemicals for inhibitory activity using clone C2, in which the M1 muscarinic receptor was linked to a NFAT beta-lactamase reporter gene readout (see Example 7). In these experiments, the inhibition measured was the inhibition of carbachol induced expression of beta-lactamase. These results, the specific inhibition of J32-6D4 cells but not C2 cells, show that the chemicals are not toxic, do not inhibit general transcription, and do not inhibit the reporter gene product.

TABLE 9.

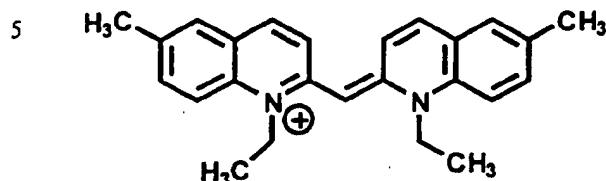
30 **Active chemicals identified as exhibiting inhibitory activity of PHA activation of clone J32-6D4**

Chemical (10 μ M)	% Inhibition of PHA activation of Clone J32-6D4	Inhibition of Clone C2	Therapeutic Category of the Chemical
Digoxin	86	+	Cardiotonic
Digitoxin	77	+	Cardiotonic
Gentian Violet	73	+	Topical anti-infective
Oxyphenbuta- zone	75	-	Anti-inflammatory
Mechloretha- mine	51	-	Anti-neoplastic
Dipyridithione	70	+	Anti-bacterial
Ouabain	50	+	Cardiotonic
Thioguanine	50	+	Anti-neoplastic

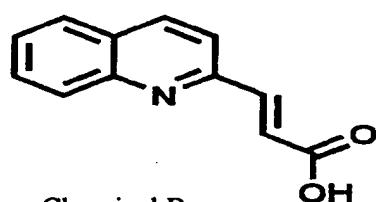
Example 10 Screening a library of structurally characterized chemicals having unknown pharmacological properties for modulating activity of the T-cell receptor pathway using a T-cell activated BLEC clone

- Having demonstrated in Example 9 that clone J32-6D4 performs robustly in a chemical screen, this clone was used to screen an additional 7,500 chemicals from a proprietary chemical library at a concentration of 10 μ M per chemical. This collection of chemicals, unlike the collection of chemicals used in Example 9, contains chemicals without known pharmacological activity. Seventy-seven chemicals showed at least 50% inhibition of PHA activation of beta-lactamase expression following the general procedures set forth in Example 7. These 77 chemicals were re-tested for this activity using the same procedure and 31 chemicals were confirmed to have activity. The IC50 values of the inhibition of PHA activation of beta-lactamase expression were determined for these 31 chemicals using concentrations of chemical between about 20 μ M to 2 nM. IC50 values reflect the concentration of a chemical needed to inhibit the PHA activation of the clone by 50% and were determined using known methods. These 31 chemicals were also tested for their cross inhibition of cabachol induced activation of beta-lactamase expression of clone C2 as described in Example 8.
- Two chemicals, designated chemical A and chemical B, exhibited an IC50 values of about 200 nM and specifically inhibited the PHA activation of beta-lactamase

expression of clone J32-6D4 but not the carbachol activation of clone C2 at the concentration tested. All of the other 31 chemicals either inhibited both clone J32-6D4 and clone C2, or had IC₅₀ values above 1 μ M.



10 Chemical A



Chemical B

Chemicals A and B were further tested for their anti-proliferative effect on Jurkat cells and mouse L-cells (mouse fibroblast cell line). Chemical B showed no anti-proliferative effect on both the Jurkats and L-cells at concentrations up to 10 μ M.

15 Chemical A exhibited an anti-proliferative effect on the Jurkats and L-cells at 100 nM. Proliferation assays were performed by seeding about 20,000 cells unactivated by PHA into a 24 well plate. These cells were contacted with chemicals and were then incubated at 37°C for five days. The cells were contacted with 10 μ g/ml of MTT (Sigma Chemical Co., MO) for three hours. The cells were then collected, resuspended in isopropanol, and 20 the absorbance was read in a plate reader at a wavelength of 570 nM with a background subtraction at a reading at a wavelength of 690 nM (see, Carmichael et al., Cancer Res. 47:936 (1987)).

Example 11 Effects of identified chemicals on primary human T-cell proliferation.

25 An assay was developed to test the chemicals identified in Example 9 for their ability to inhibit the activation and proliferation of normal peripheral white blood cells to confirm their presumptive activity (see generally, Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Press, (1988)). Peripheral blood from normal humans was drawn into heparanized Vacutainer® tubes and incubated with various 30 concentrations of (superantigen) staphylococcal enterotoxin B (SEB, at 0.001 to 10 ng/ml) for 1 hour at 37°C. Brefeldin A, which was added and the cells were incubated an additional 5 hours. EDTA was added to detach the cells, and a 100 μ L aliquot was

removed, the red blood cells lysed with ammonium chloride, the remaining cells counted and their viability determined using viability staining using known methods. The red blood cells remaining in the original sample were lysed with ammonium chloride and the remaining cells (leukocytes) were permeabilized with FACS permabilizing solution using established methods. These leukocytes were harvested by centrifugation, washed and stained with the combination of antibodies CD69, IFN- γ and CD3, which were detectably labeled. Control cells consisted of cells incubated in the absence of SEB and staining control cells consisted of cells stained with CD69/MsIgG1 and CD3 antibodies, which were detectably labeled. Similar cultures will be incubated for 71 hours, pulsed with tritiated thymidine for 1 hour and harvested and the incorporated radioactivity counted by scintillation to determine a stimulation index using established methods.

Using preferred concentrations of SEB, various concentrations of cyclosporin A (CsA) were added to determine optimal conditions of CsA for blocking of SEB stimulation of peripheral blood T-cells for use as a control for non-proliferative T-cells. Controls consisted of cells incubated with culture media in place of CsA. Control cultures incubated for 1 hour were blocked with Brefeldin A for an additional 5 hours, harvested, and stained for intracellular IFN- γ or cultured for an additional 71 hours, pulsed with tritiated thymidine for one hour, harvested, and counted by liquid scintillation.

Using preferred concentrations of SEB and CsA, blood from normal donors was stimulated in the presence and absence of CsA. This established expected normal ranges for the degree of activation (% IFN- γ + activated CD3+ cells for 6 hours), proliferation (3 H-TdR uptake at 72 hours) and CsA blocking at both time points.

Using preferred conditions, human blood was incubated with Chemical A or Chemical B at 2, 20, and 200 nM. CsA was used as a positive control for T-cell suppression. One hour cultures were blocked with Brefeldin A for an additional 5 hours, harvested and counted by liquid scintillation. Cell counts and percent viability were reported for each culture condition.

The results of these studies should demonstrate that at least one of the chemicals identified by the methods of the present invention have the predicted pharmacological activity in human cells.

5 **Example 12: Identification of genes expressed during developmental programs.**

Another use of this method is for the identification of genes expressed during various cellular processes, such as developmental biology and apoptosis. Genes involved in specific developmental programs, such as the differentiation of pre-adiposites to mature adiposites, can be identified using this method.

10 In order to practice this method, a clone library from a pre-adipose cell line such as 3T3-L1 is made using the methods generally described in Examples 10 to 12 above.

Of course, pre-adipose cells are used rather than Jurkat cells. This cell line can be reversible differentiated to mature adiposites by exposing them to dexamethasone and indomethasone (see, Hunt et al. Proc. Natl. Acad. Sci. U.S.A. 83:3786-3789 (1986)).

15 These mature adiposites can be reversibly differentiated to pre-adiposites with Tumor Necrosis Factor alpha TNFa (see, Torti et al. J. Cell. Biol. 108:1105-1113 (1989)). Thus, a cell library capable of signaling the expression of genes involved in cellular differentiation can be made.

20 The 3T3-L1 gene trap library is FACS sorted to remove blue constitutively expressing beta-lactamase cells. The remaining green cells are then differentiated into mature adiposites using the dexamethasone and indomethasone. Blue (beta-lactamase expressing) cells are isolated using FACS. These clones represent cells in which the trapping construct integrates into a gene that is expressed in differentiated adiposites, but not in undifferentiated adiposites. This process can be repeated multiple times to insure enrichment for cells that express adipose specific genes.

25 Alternatively, cell clones can be isolated which are differentiated for a specific time interval. For instance, blue and green cells differentiated for 2 days with dexamethasone and indomethasone are sorted. These populations of cells represent cells in which the trapping construct integrates into a gene that is expressed early in the

differentiation process. This allows the identification of genes that are expressed during the developmental program but are not expressed in pre-adiposites or mature adiposites. This method can be used to isolated genes expressed during a variety of developmental programs, including but not limited to neuronal, cardiac, muscle, and cancer cells.

5 These cell lines can be used to identify genes involved in the differentiation process, and can also be used to screen chemicals that modulate the differentiation process using the methods described in Examples 8 to 10 above. Drugs that can be identified include those that enhance the growth of cells, such as neuronal cells, or depress the growth or reverse differentiation of cells, such as cancer cells.

10

Example 13: Assays for modulators of G-protein coupled receptors

The general procedures of Examples 8 to 10 can be used in an analogous manner to identify cell lines suitable for screens for G-protein coupled receptors (GPCRs).
15 GPCRs are known to signal via one of several intracellular pathways. These pathways can be activated pharmacologically in cell libraries to yield potential screening cell lines. For example, Gq coupled GPCRs are known to raise intracellular free calcium via activation of phospholipase C_b (PLC_b). By isolating cell lines responsive to an increase in calcium from the genomic library (e.g. induced by ionomycin or thapsigargin), screen
20 cell lines are generated.

For example, a calcium-sensitive clone was transfected with a Gq-type GPCR by electroporation. Cells from clone J389PTI4 were transfected by electroporation with a plasmid (pcDNA3 (Invitrogen) or pcDNA3-M1 (pcDNA3 that can operably express M1 receptor) to make cell lines J389PTI4/pcDNA3 and J389PTI4/pcDNA3-M1). Cell line
25 J389PTI4/pcDNA3-M1 expressed the M1 receptor, whereas the cell line J389PTI4/pcDNA3 did not. Thus, the J389PTI4/pcDNA3 cell is a control cell. Two days after transfection, cells were stimulated with 20 μ M carbachol in 96-well microtiter plate for 6 hours in 37 °C. These cells were contacted with CCF-2 dye for another 90 minutes. The 460/530 ratio changes were measured in a Cytoflour (Series 4000 Model) (Perceptive
30 Biosystems) fluorescence plate reader and correspond to reporter gene expression. These

results are summarized in **Table 10**. The ability of the transiently-transfected clone to detect a ligand for the GPCR demonstrates the potential of generating screening cell lines using clones made following the procedures of the present invention. The stimulation by carbachol detected in the transient transfection assay represents a response in about 20% of the cells. To develop a stable screening cell line for the M1 receptor, this population can be sorted for individual clones responsive to carbachol and those clones can be expanded and screened to identify the most responsive clones.

Similar methods can be used to generate cell lines for Gs or Gi-coupled receptors. In these cases, clones responsive to increases or decreases in cAMP can be isolated. A variety of cell lines can be used for these procedures, such as CHO, HEK293, Neuroblastoma, P19, F11, and NT-2 cells.

TABLE 10
Cell lines that report modulation of the M1 receptor pathway

Cell Line	Relative expression of beta-lactamase in cells Exposed to the indicated stimuli		
	Unstimulated	30 μ M Carbachol	10 nM PHA
J389PTI4/pcDNA3	1	1	12
J389PTI4/pcDNA3-M1	1	4	13

15

Publications

Articles

- G. Friedrich, P. Soriano, Methods in Enzymology, Vol. 225: 681 (1993)
- 20 G. Friedrich, P. Soriano, Genes & Development, Vol. 5: 1513 (1991)
- A. Gossler, et al., Reports, 28 April: 463 (1989)
- D. Hill, W. Wurst, Methods in Enzymology, Vol. 225: 664 (1993)
- P. Mountford, A. Smith, TIG, Vol. 11 No. 5: 179 (1995)
- P. Mountford, et al., Proc. Natl. Acad. Sci. USA, Vol. 91: 4303 (1994)
- 25 Q. Niwa, et al., J. Biochem, Vol. 13: 343 (1993)
- R. Reddy, et al., Proc. Natl. Acad. Sci. USA, Vol. 89: 6721 (1992)

S. Shapiro, P. Senapathy, Nucleic Acids Research, Vol. 17, No. 17: 7155 (1987)

T. Skarnes, et al., Genes & Development, Vol. 6: 903 (1992)

W. Wurst, et al., Genetics, Vol. 139: 889 (1995)

All publications, including patent documents and scientific articles, referred to in
5 this application are incorporated by reference in their entirety for all purposes to the same
extent as if each individual publication were individually incorporated by reference.

All headings are for the convenience of the reader and should not be used to limit
the meaning of the text that follows the heading, unless so specified.

SEQUENCE ID. LISTING

SEQ. ID NO. 1: range 1 to 795

79

	10	20	30	40	50
5	*	*	*	*	*
	ATG AGT CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG				
	Met Ser His Pro Glu Thr Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu				
10	60	70	80	90	100
	*	*	*	*	*
	GGT GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT				
	Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu				
	110	120	130	140	150
15	*	*	*	*	*
	GAG AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT				
	Glu Ser Phe Arg Pro Glu Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val				
	160	170	180	190	200
	*	*	*	*	*
20	CTG CTA TGT GGC GCG GTA TTA TCC CGT GTT GAC GCC GGG CAA GAG CAA CTC				
	Leu Leu Cys Gly Ala Val Leu Ser Arg Val Asp Ala Gly Gln Glu Gln Leu				
	210	220	230	240	250
	*	*	*	*	*
	GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC				
25	Gly Arg Arg Ile His Tyr Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val				
	260	270	280	290	300
	*	*	*	*	*
	ACA GAA AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT				
	Thr Glu Lys His Leu Thr Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala				
30	310	320	330	340	350
	*	*	*	*	*
	GCC ATA ACC ATG AGT GAT AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC				
	Ala Ile Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu Thr Thr Ile				
	360	370	380	390	400
35	*	*	*	*	*
	GGA GGA CCG AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA				
	Gly Gly Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val				
	410	420	430	440	450
	*	*	*	*	*
40	ACT CGC CTT GAT CGT TGG GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC				
	Thr Arg Leu Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp				
	460	470	480	490	500
	*	*	*	*	*
	GAG CGT GAC ACC ACG ATG CCT GCA GCA ATG GCA ACA ACG TTG CGC AAA CTA				
45	Glu Arg Asp Thr Thr Met Pro Ala Ala Met Ala Thr Thr Leu Arg Lys Leu				
	520	530	540	550	560
	*	*	*	*	*
	TTA ACT GGC GAA CTA CTT ACT CTA GCT TCC CGG CAA CAA TTA ATA GAC TGG				
	Leu Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp				
50	570	580	590	600	610
	*	*	*	*	*
	ATG GAG GCG GAT AAA GTT GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT				

Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro Ala
 620 630 640 650 660
 * * * * * * * * * * * * *
 GGC TGG TTT ATT GCT GAT AAA TCT GGA GCC GGT GAG CGT GGG TCT CGC GGT
 5 Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser Arg Gly
 670 680 690 700 710
 * * * * * * * * * * * * *
 ATC ATT GCA GCA CTG GGG CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC
 Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile
 10 720 730 740 750 760
 * * * * * * * * * * * * *
 TAC ACG ACG GGG AGT CAG GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT
 Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn Arg Gln Ile Ala
 770 780 790
 15 * * * * * * * * * * * *
 GAG ATA GGT GCC TCA CTG ATT AAG CAT TGG
 Glu Ile Gly Ala Ser Leu Ile Lys His Trp

20 SEQ.ID NO. 2: range 1 to 858

	10	20	30	40	50
	*	*	*	*	*
25	ATG AGA ATT CAA CAT TTC CGT GTC GCC CTT ATT CCC TTT TTT GCG GCA TTT				
	Met Arg Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala Phe				
	60 60	70 70	80 80	90 90	100 100
	*	*	*	*	*
30	TGC CTT CCT GTT TTT GGT CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT				
	Cys Leu Pro Val Phe Gly His Pro Glu Thr Leu Val Lys Val Lys Asp Ala				
	110 110	120 120	130 130	140 140	150 150
	*	*	*	*	*
	GAA GAT CAG TTG GGT GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC				
	Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser				
35	160 160	170 170	180 180	190 190	200 200
	*	*	*	*	*
	GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC				
	Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe Pro Met Met Ser				
	210 210	220 220	230 230	240 240	250 250
40	*	*	*	*	*
	ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA TTA TCC CGT GTT GAC GCC GGG				
	Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser Arg Val Asp Ala Gly				
	260 260	270 270	280 280	290 290	300 300
	*	*	*	*	*
45	CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT GAG				
	Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser Gln Asn Asp Leu Val Glu				
	310 310	320 320	330 330	340 340	350 350
	*	*	*	*	*
	TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA				
50	Tyr Ser Pro Val Thr Glu Lys His Leu Thr Asp Gly Met Thr Val Arg Glu				
	360 360	370 370	380 380	390 390	400 400
	*	*	*	*	*
	TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT AAC ACT GCG GCC AAC TTA CTT				

Leu Cys Ser Ala Ala Ile Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu
 410 420 430 440 450
 * * * * * * * * * * * * * *
 CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG
 5 Leu Thr Thr Ile Gly Gly Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met
 460 470 480 490 500 510
 * * * * * * * * * * * * * *
 GGG GAT CAT GTA ACT CGC CTT GAT CGT TGG GAA CCG GAG CTG AAT GAA GCC
 Gly Asp His Val Thr Arg Leu Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala
 10 520 530 540 550 560
 * * * * * * * * * * * * * *
 ATA CCA AAC GAC GAG CGT GAC ACC ACG ATG CCT GCA GCA ATG GCA ACA ACG
 Ile Pro Asn Asp Glu Arg Asp Thr Thr Met Pro Ala Ala Met Ala Thr Thr
 570 580 590 600 610
 15 * * * * * * * * * * * * * *
 TTG CGC AAA CTA TTA ACT GGC GAA CTA CTT ACT CTA GCT TCC CGG CAA CAA
 Leu Arg Lys Leu Leu Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln
 620 630 640 650 660
 * * * * * * * * * * * * * *
 20 TTA ATA GAC TGG ATG GAG GCG GAT AAA GTT GCA GGA CCA CTT CTG CGC TCG
 Leu Ile Asp Trp Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser
 670 680 690 700 710
 * * * * * * * * * * * * * *
 GCC CTT CCG GCT GGC TGG TTT ATT GCT GAT AAA TCT GGA GCC GGT GAG CGT
 25 Ala Leu Pro Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg
 720 730 740 750 760
 * * * * * * * * * * * * * *
 GGG TCT CGC GGT ATC ATT GCA GCA CTG GGG CCA GAT GGT AAG CCC TCC CGT
 Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg
 30 770 780 790 800 810
 * * * * * * * * * * * * * *
 ATC GTA GTT ATC TAC ACG ACG GGG AGT CAG GCA ACT ATG GAT GAA CGA AAT
 Ile Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn
 820 830 840 850
 35 * * * * * * * * * * * * * *
 AGA CAG ATC GCT GAG ATA GGT GCC TCA CTG ATT AAG CAT TGG
 Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp

40

SEQ. ID NO. 3: range 1 to 795

45 AAGCTTTGCAGAACGCTCAGAATAACGCAACTTCCGGTACCACC
 10 20 30 40 50
 * * * * * * * * * * * * * *
 *
 ATG GGG CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT
 50 GCA
 Met Gly His Pro Glu Thr Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly
 Ala
 60 70 80 90 100

CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT
 Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser
 110 120 130 140 150
 *
 TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA
 Phe Arg Pro Glu Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu
 160 170 180 190 200 210
 *
 10 TGT GGC GCG GTA TTA TCC CGT GAT GAC GCC GGG CAA GAG CAA CTC GGT CGC
 Cys.Gly Ala Val Leu Ser Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg
 220 230 240 250 260
 *
 CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA
 15 Arg Ile His Tyr Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu
 270 280 290 300 310
 *
 AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA
 Lys His Leu Thr Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile
 20 320 330 340 350 360
 *
 ACC ATG AGT GAT AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA
 Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu Thr Thr Ile Gly Gly
 370 380 390 400 410
 *
 25 CCG AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT CGC
 Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg
 420 430 440 450 460
 *
 30 CTT GAT CAT TGG GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT
 Leu Asp His Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg
 470 480 490 500 510
 *
 35 GAC ACC ACG ATG CCT GTA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA ACT
 Asp Thr Thr Met Pro Val Ala Met Ala Thr Thr Leu Arg Lys Leu Leu Thr
 520 530 540 550 560
 *
 GGC GAA CTA CTT ACT CTA GCT TCC CGG CAA CAA TTA ATA GAC TGG ATG GAG
 Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp Met Glu
 40 570 580 590 600 610
 *
 GCG GAT AAA GTT GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT GGC TGG
 Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro Ala Gly Trp
 620 630 640 650 660
 *
 45 TTT ATT GCT GAT AAA TCT GGA GCC GGT GAG CGT GGG TCT CGC GGT ATC ATT
 Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser Arg Gly Ile Ile
 670 680 690 700 710 720
 *
 50 GCA GCA CTG GGG CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC TAC ACG
 Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile Tyr Thr
 730 740 750 760 770

ACG GGG AGT CAG GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT GAG ATA
 Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn Arg Gln Ile Ala Glu Ile
 780 790

5 GGT GCC TCA CTG ATT AAG CAT TGG
 Gly Ala Ser Leu Ile Lys His Trp

10 SEQ.ID NO. 4: range 1 to 792

	10	20	30	40	50
15	*	*	*	*	*
	ATG GAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT	Met Asp Pro Glu Thr Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly			
	60	70	80	90	100
20	*	*	*	*	*
	GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG	Ala Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu			
	110	120	130	140	150
25	*	*	*	*	*
	AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG	Ser Phe Arg Pro Glu Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu			
	160	170	180	190	200
	*	*	*	*	*
	CTA TGT GGC GCG GTA TTA TCC CGT ATT GAC GCC GGG CAA GAG CAA CTC GGT				
30	Leu Cys Gly Ala Val Leu Ser Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly				
	210	220	230	240	250
	*	*	*	*	*
	CGC CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA				
	Arg Arg Ile His Tyr Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr				
35	260	270	280	290	300
	*	*	*	*	*
	GAA AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC				
	Glu Lys His Leu Thr Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala				
	310	320	330	340	350
40	*	*	*	*	*
	ATA ACC ATG AGT GAT AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA				
	Ile Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly				
	360	370	380	390	400
	*	*	*	*	*
45	GGA CCG AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT				
	Gly Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr				
	410	420	430	440	450
	*	*	*	*	*
	CGC CTT GAT CAT TGG GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG				
50	Arg Leu Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu				
	460	470	480	490	500
	*	*	*	*	*
	CGT GAC ACC ACG ATG CCT GTA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA				

	Arg	Asp	Thr	Thr	Met	Pro	Val	Ala	Met	Ala	Thr	Thr	Leu	Arg	Lys	Leu	Leu
					520		530		540		550		560				
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
5	ACT	GGC	GAA	CTA	CTT	ACT	CTA	GCT	TCC	CGG	CAA	CAA	TTA	ATA	GAC	TGG	ATG
	Thr	Gly	Glu	Leu	Leu	Thr	Leu	Ala	Ser	Arg	Gln	Gln	Leu	Ile	Asp	Trp	Met
	570		580		590		600		610								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
10	GAG	GCG	GAT	AAA	GTT	GCA	GGA	CCA	CTT	CTG	CGC	TCG	GCC	CTT	CCG	GCT	GGC
	Glu	Ala	Asp	Val	Ala	Gly	Pro	Leu	Leu	Arg	Ser	Ala	Leu	Pro	Ala	Gly	
	620		630		640		650		660								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
15	TGG	TTT	ATT	GCT	GAT	AAA	TCT	GGA	GCC	GGT	GAG	CGT	GGG	TCT	CGC	GGT	ATC
	Trp	Phe	Ile	Ala	Asp	Lys	Ser	Gly	Ala	Gly	Glu	Arg	Gly	Ser	Arg	Gly	Ile
	670		680		690		700		710								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
20	ATT	GCA	GCA	CTG	GGG	CCA	GAT	GGT	AAG	CCC	TCC	CGT	ATC	GTA	GTT	ATC	TAC
	Ile	Ala	Ala	Leu	Gly	Pro	Asp	Gly	Lys	Pro	Ser	Arg	Ile	Val	Val	Ile	Tyr
	720		730		740		750		760								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
25	ACG	ACG	GGG	AGT	CAG	GCA	ACT	ATG	GAT	GAA	CGA	AAT	AGA	CAG	ATC	GCT	GAG
	Thr	Thr	Gly	Ser	Gln	Ala	Thr	Met	Asp	Glu	Arg	Asn	Arg	Gln	Ile	Ala	Glu
	770		780		790												
*	*	*	*	*	*												
	ATA	GGT	GCC	TCA	CTG	ATT	AAG	CAT	TGG								
	Ile	Gly	Ala	Ser	Leu	Ile	Lys	His	Trp								

30
SEQ. ID NO. 5: range 1 to 786

	10	20	30	40	50												
35	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	ATG	AAA	GAT	GAT	TTT	GCA	AAA	CTT	GAG	GAA	CAA	TTT	GAT	GCA	AAA	CTC	GGG
	Met	Lys	Asp	Asp	Phe	Ala	Lys	Leu	Glu	Glu	Gln	Phe	Asp	Ala	Lys	Leu	Gly
	60		70		80		90		100								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
40	ATC	TTT	GCA	TTG	GAT	ACA	GGT	ACA	AAC	CGG	ACG	GTA	GCG	TAT	CGG	CCG	GAT
	Ile	Phe	Ala	Leu	Asp	Thr	Gly	Thr	Asn	Arg	Thr	Val	Ala	Tyr	Arg	Pro	Asp
	110		120		130		140		150								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
45	GAG	CGT	TTT	GCT	TTT	GCT	TCG	ACG	ATT	AAG	GCT	TTA	ACT	GTA	GGC	GTG	CTT
	Glu	Arg	Phe	Ala	Phe	Ala	Ser	Thr	Ile	Lys	Ala	Leu	Thr	Val	Gly	Val	Leu
	160		170		180		190		200								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
50	TTG	CAA	CAG	AAA	TCA	ATA	GAA	GAT	CTG	AAC	CAG	AGA	ATA	ACA	TAT	ACA	CGT
	Leu	Gln	Gln	Lys	Ser	Ile	Glu	Asp	Leu	Asn	Gln	Arg	Ile	Thr	Tyr	Thr	Arg
	210		220		230		240		250								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	GAT	GAT	CTT	GTA	AAC	TAC	AAC	CCG	ATT	ACG	GAA	AAG	CAC	GTT	GAT	ACG	GGA
	Asp	Asp	Leu	Val	Asn	Tyr	Asn	Pro	Ile	Thr	Glu	Lys	His	Val	Asp	Thr	Gly

	260	270	280	290	300	
	*	*	*	*	*	*
	ATG ACG CTC AAA GAG CTT GCG GAT GCT TCG CTT CGA TAT AGT GAC AAT GCG	Met Thr Leu Lys Glu Leu Ala Asp Ala Ser Leu Arg Tyr Ser Asp Asn Ala				
5	310	320	330	340	350	
	*	*	*	*	*	*
	GCA CAG AAT CTC ATT CTT AAA CAA ATT GGC GGA CCT GAA AGT TTG AAA AAG	Ala Gln Asn Leu Ile Leu Lys Gln Ile Gly Gly Pro Glu Ser Leu Lys Lys				
	360	370	380	390	400	
10	*	*	*	*	*	*
	GAA CTG AGG AAG ATT GGT GAT GAG GTT ACA AAT CCC GAA CGA TTC GAA CCA	Glu Leu Arg Lys Ile Gly Asp Glu Val Thr Asn Pro Glu Arg Phe Glu Pro				
	410	420	430	440	450	
	*	*	*	*	*	*
15	GAG TTA AAT GAA GTG AAT CCG GGT GAA ACT CAG GAT ACC AGT ACA GCA AGA	Glu Leu Asn Glu Val Asn Pro Gly Glu Thr Gln Asp Thr Ser Thr Ala Arg				
	460	470	480	490	500	510
	*	*	*	*	*	*
	GCA CTT GTC ACA AGC CTT CGA GCC TTT GCT CTT GAA GAT AAA CTT CCA AGT	Ala Leu Val Thr Ser Leu Arg Ala Phe Ala Leu Glu Asp Lys Leu Pro Ser				
20	520	530	540	550	560	
	*	*	*	*	*	*
	GAA AAA CGC GAG CTT TTA ATC GAT TGG ATG AAA CGA AAT ACC ACT GGA GAC	Glu Lys Arg Glu Leu Leu Ile Asp Trp Met Lys Arg Asn Thr Thr Gly Asp				
25	570	580	590	600	610	
	*	*	*	*	*	*
	GCC TTA ATC CGT GCC GGA GCG GCA TCA TAT GGA ACC CGG AAT GAC ATT GCC	Ala Leu Ile Arg Ala Gly Val Pro Asp Gly Trp Glu Val Ala Asp Lys Thr				
	620	630	640	650	660	
30	*	*	*	*	*	*
	ATC ATT TGG CCG CCA AAA GGA GAT CCT GTC GGT GTG CCG GAC GGT TGG GAA	Gly Ala Ala Ser Tyr Lys Gly Asp Pro Val Gly Thr Arg Asn Asp Ile Ala				
	670	680	690	700	710	
	*	*	*	*	*	*
35	GTG GCT GAT AAA ACT GTT CTT GCA GTA TTA TCC AGC AGG GAT AAA AAG GAC	Ile Ile Trp Pro Pro Val Leu Ala Val Leu Ser Ser Arg Asp Lys Lys Asp				
	720	730	740	750	760	
	*	*	*	*	*	*
	GCC AAG TAT GAT GAT AAA CTT ATT GCA GAG GCA ACA AAG GTG GTA ATG AAA	Ala Lys Tyr Asp Asp Lys Leu Ile Ala Glu Ala Thr Lys Val Val Met Lys				
40	770	780				
	*	*	*	*	*	*
	GCC TTA AAC ATG AAC GGC AAA	Ala Leu Asn Met Asn Gly Lys				
45						

We claim:

1. A method for identifying proteins or chemicals that directly or indirectly modulate a genomic polynucleotide comprising:
providing β -lactamase integrated into a non-yeast, eukaryotic genome contained
5 in at least one living cell,
contacting said cell with a predetermined concentration of a modulator, and
detecting BL activity from said cell.
2. The method of claim 1, wherein said detecting further comprises measuring cleavage of a membrane permeant BL substrate, wherein said membrane permeant BL substrate is transformed in said cell.
10
3. The method of claim 2, wherein said membrane permeant BL substrate comprises a donor and acceptor.
15
4. The method of claim 3, wherein said detecting further comprises measuring FRET between said donor and said acceptor.
5. The method of claim 2, wherein said living cell is a mammalian cell.
15
6. The method of claim 5, wherein said BL expression construct randomly integrates into said genome.
20
7. The method of claim 6, wherein said living cell is contacted with said modulator prior to inserting of said BL expression construct in said non-yeast, eukaryotic genome and further comprising the step of determining the coding nucleic acid sequence of a polynucleotide operably linked to said BL expression construct, wherein said construct comprises a splice donor, a splice acceptor and an IRES element.
25
8. The method of claim 5, wherein said BL expression construct encodes cytosolic BL and said cell comprises a receptor that is known to bind said modulator.
9. The method of claim 8, wherein said receptor is a nuclear receptor heterologously expressed by said cell.
25
10. The method of claim 8, wherein said receptor has a transmembrane domain and is homologously expressed by said cell.
11. The method of claim 10, wherein said modulator is a non-peptide.

12. The method of claim 8, wherein said cell is contacted with a predetermined concentration of a second modulator and detecting BL activity before and after contacting said cell with said second modulator.
13. The method of claim 5, wherein said cell comprises an orphan protein heterologously expressed by said cell.
14. The method of claim 5, wherein said BL activity is increased in the presence of said modulator compared with the β -lactamase activity in the absence of said modulator.
15. The method of claim 5, wherein said modulator is known to bind to a receptor expressed by said cell and said BL activity in said cell is increased in the presence of said modulator compared to the BL activity detected from a corresponding cell in the presence of said modulator, wherein said corresponding cell does not express of said receptor.
16. A method of identifying active genomic polynucleotides, comprising:
 - contacting living cells with a membrane permeant BL substrate, and
 - sorting living cells by fluorescence,

wherein said cells are eukaryotic cells and comprise a genome having a stably integrated BL expression construct and said fluorescence indicates BL activity.
17. The method of claim 16, wherein said sorting further comprises measuring cleavage of a membrane permeant BL substrate by fluorescence spectroscopy in a FACS, wherein said membrane permeant BL substrate is transformed in said cell.
18. The method of claim 17, wherein said membrane permeant substrate BL substrate has a donor and acceptor and said measuring further comprises measuring FRET between a donor and an acceptor.
19. The method of claim 17, wherein said sorting further comprises separating said cells without BL activity from said cells with BL activity.
20. The method of claim 19, wherein said cells are contacted with only a cell culture medium in the absence of a test chemical.
21. The method of claim 20, wherein said cells without BL activity are contacted with a test chemical and further sorted by fluorescence for BL activity.
22. The method of claim 21, wherein said test chemical is an agonist.

23. The method of claim 21, wherein said test chemical is an antagonist.
24. The method of claim 21, wherein said cells without BL activity are contacted with a test chemical and further sorted by fluorescence for BL activity.
25. The method of claim 22, wherein said cells with BL activity are contacted with an antagonist and further sorted by fluorescence for BL activity.
5
26. The method of claim 17, wherein said cells express an identified receptor that binds a modulator known to bind to said identified receptor.
27. The method of claim 26, wherein said living cells comprise a heterologous G-protein.
10
28. The method of claim 17, wherein said living cells comprise a heterologous protein having a membrane domain.
15
29. A composition of matter comprising a non-yeast, eukaryotic cell having a genome with a stably integrated BL expression construct comprising a polynucleotide encoding a protein having BL activity, an IRES element, a splice donor site and a splice acceptor site.
15
30. The composition of matter of claim 29, further comprising a heterologous protein expressed in said cell.
31. The composition of matter of claim 30, wherein said cell is a mammalian cell.
32. The composition of matter of claim 31, wherein said polynucleotide contains nucleic acid sequences that are preferred by said mammalian cell for expression.
20
33. The composition of matter of claim 32, wherein said cell further comprises a membrane permeant BL substrate, wherein said membrane permeant BL substrate is transformed inside said cell by intracellular esterases.
34. The composition of matter of claim 33, wherein said polynucleotide encodes a cytosolic BL.
25
35. A method of screening compounds with an active genomic polynucleotide, comprising:
 - 1) optionally contacting a multiclonal population of cells with a first test chemical prior to separating said cells by a FACS,

- 2) separating by a FACS said multiclonal population of cells into BL expressing cells and non-BL expressing cells, wherein said BL expressing cells have a detectable difference in cellular fluorescence properties compared to non-BL expressing cells, and
- 5 Ai) contacting said non-BL expressing cells with a second test chemical, and
 Aii) sorting by a FACS said non-BL expressing cells into a) second test chemical activated cells and b) second test chemical non-activated cells,
 wherein said second test chemical activated cells have BL activity detectable by a FACS and said second test chemical non-activated cells have no BL activity detectable by FACS, or
- 10 Bi) contacting said BL expressing cells with a third test chemical, and
 Bii) sorting by a FACS said BL expressing cells into a) third test chemical activated cells and b) third test chemical non-activated cells,
 wherein said third test chemical activated cells have BL activity detectable by a FACS and said third test chemical non-activated cells have no BL activity detectable by FACS,
15 wherein said multiclonal population of cells comprises eukaryotic cells having a BL expression construct integrated into a genome of said eukaryotic cells and a membrane permanent BL substrate transformed inside said cells to a membrane impermeant BL substrate.
- 20 36. The method of claim 35, wherein said BL activity is measured by FRET.
 37. The method of claim 35, wherein said steps of Ai and Aii or Bi and Bii are repeated.
 38. The method of claim 35, wherein said second test chemical activated cells are washed, then contacted with a modulator in the presence of said second test chemical and tested for BL activity.
25 39. The method of claim 38, wherein said modulator is present in a concentration of 10⁻¹² M or less.
 40. The method of claim 35, wherein said eukaryotic cells express a heterologous protein.

41. A method for identifying an expressed protein that directly or indirectly modulates a genomic polynucleotide, comprising: providing at least one living non-yeast, eukaryotic cell comprising a β -lactamase polynucleotide that can be under transcriptional control of said at least one living non-yeast, eukaryotic cell's genome and stably integrated into a genomic polynucleotide site, contacting said cell with a predetermined concentration of a known modulator, and detecting β -lactamase activity from said at least one living non-yeast, eukaryotic cell; wherein said at least one living non-yeast, eukaryotic cell expresses a heterologous protein and said known modulator increases or decreases the expression of said β -lactamase polynucleotide in the presence of said heterologous protein.
42. The method of claim 41, wherein said detecting further comprises measuring cleavage of a membrane permeant β -lactamase substrate, wherein said membrane permeant β -lactamase substrate is transformed in said at least one living non-yeast, eukaryotic cell.
43. The method of claim 42, wherein said membrane permeant β -lactamase substrate has a donor and acceptor in said at least one living non-yeast, eukaryotic cell.
44. The method of claim 3, wherein said method further comprises sorting a population of cells with a FACS.
45. The method of claim 41, wherein said cell is a mammalian cell.
46. The method of claim 45, wherein said β -lactamase polynucleotide includes a β -lactamase expression construct for random integration into said genome.
47. The method of claim 46, further comprising the step of determining a portion of the coding nucleic acid sequence of a polynucleotide operably linked to said β -lactamase expression construct.
48. The method of claim 45, wherein said BL expression construct comprises cytosolic β -lactamase, said construct comprises a splice donor, a splice acceptor and an IRES element and said cell comprises a receptor that is known to bind said known modulator.

49. The method of claim 45, wherein said heterologous protein is selected from the group consisting of hormone receptors, intracellular receptors, receptors of the cytokine superfamily, G-protein coupled receptors, heterologous G-proteins, neurotransmitter receptors, and tyrosine kinase receptors.
- 5 50. The method of claim 45, wherein said heterologous protein has a transmembrane domain.
51. The method of claim 50, further comprising over expressing said heterologous protein.
52. The method of claim 45, wherein said at least one living non-yeast, eukaryotic 10 cell is contacted with a predetermined concentration of a second modulator and detecting β-lactamase activity after contacting said cell with said known modulator.
53. The method of claim 45, wherein said cell comprises an orphan protein heterologously expressed by said at least one living non-yeast, eukaryotic cell.
54. The method of claim 45, wherein said β-lactamase activity is increased in the 15 presence of said modulator compared to the absence of said modulator.
55. The method of claim 45, wherein said known modulator is known to bind to a receptor and said β-lactamase activity in said at least one living non-yeast, eukaryotic cell is increased in the presence of said modulator compared to the β-lactamase activity detected from a corresponding cell in the presence of said known modulator, wherein said 20 corresponding cell does not express said heterologous protein.
56. A method for identifying modulators, comprising:
- a) contacting at least one living mammalian cell with a test chemical at a predetermined concentration and a known modulator at a predetermined concentration, wherein said at least one living mammalian cell comprises a β-lactamase polynucleotide that can be under transcriptional control of said at least 25 one living mammalian cell's genome and stably integrated into a genomic polynucleotide site, and
- b) detecting expression of said β-lactamase polynucleotide by said at least one living mammalian cell, wherein said known modulator increases or decreases

- expression of said β -lactamase polynucleotide located at said genomic polynucleotide site.
57. The method of claim 56, wherein said test chemical changes expression of said β -lactamase polynucleotide by said known modulator.
- 5 58. The method of claim 56, wherein said β -lactamase polynucleotide further comprises a splice acceptor site.
59. The method of claim 58, wherein said β -lactamase polynucleotide further comprises an IRES.
60. The method of claim 57, wherein said test chemical or known modulator is provided at a concentration less than about 1 μ M.
- 10 61. The method of claim 56, further comprising separating a population of living mammalian cells into 1) a population of living mammalian cells that expresses β -lactamase and 2) a population of living mammalian cells that does not express β -lactamase.
- 15 62. The method of claim 61, wherein said separating further comprises measuring cleavage of a membrane permeant β -lactamase substrate in said population of living mammalian cells by fluorescence spectroscopy in a FACS, wherein the fluorescence of said membrane permeant β -lactamase substrate is transformed by β -lactamase in at least one living mammalian cell.
- 20 63. The method of claim 56, wherein said known modulator modulates a receptor selected from the group consisting of intracellular receptors and G-protein coupled receptors.
64. The method of claim 63, wherein said known modulator is an agonist.
65. The method of claim 63, wherein said known modulator is an antagonist.
- 25 66. The method of claim 64, wherein said known modulator is contacted with said at least one living mammalian cell prior to contacting said test chemical with said at least one living mammalian cell.
67. The method of claim 56, wherein said test chemical is a modulator for a protein selected from the group consisting of hormone receptors, intracellular receptors, receptors

of the cytokine superfamily, G-protein coupled receptors, heterologous G-proteins, neurotransmitter receptors, and tyrosine kinase receptors.

68. The method of claim 56, wherein said at least one living mammalian cell further comprises a heterologously expressed protein selected from the group consisting of
5 hormone receptors, intracellular receptors, signaling molecules, receptors of the cytokine superfamily, G-protein coupled receptors, heterologous G-proteins, neurotransmitters, and tyrosine kinase receptors.
69. The method of claim 68, wherein said heterologously expressed protein is a G-protein coupled receptor or a heterologous G-protein.
- 10 70. The method of claim 56, further comprising the step of activating said at least one living mammalian cell with a G-protein coupled receptor modulator.
71. The method of claim 70, wherein said at least one living mammalian cell further comprises an orphan receptor.
72. The method of claim 56, wherein said at least one living mammalian cell is of cell
15 type from a panel of different cell types and steps (a) and (b) are performed on each cell type.
73. The method of claim 56, wherein said genomic polynucleotide site is part of a gene not known to be modulated by said known modulator.
74. The method of claim 73, wherein said known modulator is as an agonist.
- 20 75. The method of claim 74, wherein said test chemical is an antagonist.
76. The method of claim 73, wherein said known modulator is an antagonist.
77. The method of claim 76, wherein said test chemical is an agonist.
78. A method for identifying a modulator, comprising:
25 a) contacting a population of non-yeast, eukaryotic cells with a test chemical and a known modulator, wherein said population of non-yeast, eukaryotic cells comprises a genome with a stably integrated β -lactamase expression construct, comprising:
1) a polynucleotide encoding a protein having β -lactamase activity, and
2) a splice acceptor site; and

- b) detecting the activity of said β -lactamase polynucleotide expressed by said population of non-yeast, eukaryotic cells, wherein said known modulator increases or decreases the expression of said polynucleotide encoding a protein having β -lactamase activity, and said known modulator modulates a biological process or target.
- 5 79. The method of claim 78, wherein said β -lactamase expression construct further comprises a splice donor site.
80. The method of claim 79, wherein said β -lactamase expression construct further comprises an IRES element.
- 10 81. The method of claim 78, wherein said population of non-yeast, eukaryotic cells further comprises an expressed heterologous G-protein coupled receptor.
82. The method of claim 81, wherein said population of non-yeast, eukaryotic cells further comprises an orphan G-protein coupled receptor.
- 15 83. A method for identifying a ligand of a target, comprising: contacting a eukaryotic cell with a test chemical at a predetermined concentration, wherein said eukaryotic cell comprises 1) a genomic polynucleotide with a β -lactamase expression construct under expression control by a first polynucleotide in said genomic polynucleotide and 2) a target that does not normally modulate transcription of a gene product under expression control of said first polynucleotide with proviso that said target can directly or indirectly alter expression of said β -lactamase expression construct under expression control by said first polynucleotide, and
- 20 determining expression of said β -lactamase expression construct.
84. The method of claim 83, wherein said eukaryotic cell is a mammalian cell.
85. The method of claim 84, wherein said target is a heterologously expressed protein.
- 25 86. The method of claim 85, wherein said heterologously expressed protein is a membrane protein.
87. The method of claim 84, wherein said heterologously expressed protein is a GPCR.
88. The method of claim 84, wherein said heterologously expressed protein is an ion channel.

89. The method of claim 84, further comprising contacting a eukaryotic cell with a test chemical at a predetermined concentration, wherein said eukaryotic cell comprises 1) a genomic polynucleotide with a β -lactamase expression construct under expression control by a first polynucleotide in said genomic polynucleotide and 2) a target that does not normally modulate transcription of a gene product under expression control of said first polynucleotide.
- 5 90. The method of claim 84, wherein said gene product is normally expressed in a first tissue and said target is normally expressed in a second tissue, wherein said first tissue is of a different embryonic origin than said second tissue.
- 10 91. The method of claim 84, wherein said gene product is normally expressed in a first cell *in vivo* and said target is normally expressed in a second cell *in vivo*, wherein said first cell is a different cell type than said second cell.
- 15 92. The method of claim 84, wherein expression of said gene product is normally repressed and said target does not increase expression of said gene product *in vivo* in naturally occurring cells.
93. The method of claim 84, wherein said gene product is normally expressed in a first cell *in vivo* and said target is normally expressed in a second cell *in vivo*, wherein said first cell is a different cell type than said second cell.
- 20 94. The method of claim 84, wherein expression of said gene product in said eukaryotic cell is not detectable in the absence of said target and said eukaryotic cell does not express detectable levels of protein of said target in the absence of heterologous expression of said target.
95. The method of claim 84, wherein native protein of said gene product and native protein of said target are not expressed in detectable levels in a single, naturally occurring-cell.
- 25 96. The method of claim 84, wherein native protein of said target in a naturally occurring cell does not modulate expression of native protein of said gene product in said naturally occurring cell.
97. A method for identifying a cellular function of an orphan protein, comprising:

contacting a eukaryotic cell with a test chemical at a predetermined concentration, wherein said eukaryotic cell comprises 1) a genomic polynucleotide with a β -lactamase expression construct under expression control by a first polynucleotide in said genomic polynucleotide and 2) an orphan protein,

- 5 determining expression of said β -lactamase expression construct, and
identifying the function of said genomic polynucleotide with said β -lactamase expression construct or its corresponding gene where said β -lactamase expression construct has integrated.

98. The method of claim 97, wherein said eukaryotic cell is a mammalian cell.
10 99. The method of claim 98, wherein said orphan is a heterologously expressed protein.
100. The method of claim 99, wherein said heterologously expressed orphan protein has putative transmembrane domain.
101. The method of claim 98, wherein said heterologously expressed orphan protein is homologous to a GPCR of known function and is overexpressed.
15 102. A method for identifying a modulator of an orphan protein, comprising:
contacting a eukaryotic cell with a test chemical at a predetermined concentration, wherein said eukaryotic cell comprises 1) a genomic polynucleotide with a β -lactamase expression construct under expression control by a first polynucleotide in said genomic polynucleotide and 2) a orphan protein that modulates expression of said β -lactamase expression construct, and
20 determining expression of said β -lactamase expression construct.
103. The method of claim 102, wherein said eukaryotic cell is a mammalian cell.
104. The method of claim 103, wherein said orphan protein is a heterologously expressed protein.
25 105. The method of claim 102, wherein said heterologously expressed orphan protein has putative transmembrane domain.
106. The method of claim 102, wherein said heterologously expressed orphan protein is over expressed and is homologous to a GPCR of known function.
107. A method for identifying intracellular pathways, comprising:

- expressing a protein of interest in a plurality of eukaryotic cells, wherein each eukaryotic cell comprises a genomic polynucleotide with a β -lactamase expression construct under expression control by a polynucleotide in said genomic polynucleotide, and said plurality of cells has a plurality of integration sites where said β -lactamase expression construct has integrated into said genome of each said eukaryotic cell,
5 optionally contacting said plurality of eukaryotic cells with a ligand of said protein of interest,
determining expression from said β -lactamase expression construct, and
identifying said polynucleotide if said expressing of said protein of interest alters
10 expression from said β -lactamase expression construct or if said contacting said ligand of said protein of interest alters expression from said β -lactamase expression construct,
wherein alteration of said expression from said β -lactamase expression construct indicates participation of said protein of interest in an intracellular signaling pathway.
108. The method of claim 107, wherein said eukaryotic cell is a mammalian cell.
110. The method of claim 108, wherein said protein of interest is a heterologously expressed protein and has a known ligand.
111. The method of claim 109, further comprising isolating a eukaryotic cell from said plurality of eukaryotic cells and characterizing said polynucleotide.
112. The method of claim 109, wherein each said eukaryotic cell in said plurality of eukaryotic cells is an isolated, clonal population of cells.
113. The method of claim 112, wherein said plurality of cells comprises at least 10,000 isolated clonal populations of cells.
- 25 114. A method for determining a cellular response profile for a target, comprising:
expressing a protein of interest in a plurality of eukaryotic cells, wherein each eukaryotic cell comprises a genomic polynucleotide with a β -lactamase expression construct under expression control by a polynucleotide in said genomic polynucleotide,

and said plurality of cells has a plurality of integration sites where said β -lactamase expression construct has integrated into said genome of each said eukaryotic cell,

optionally contacting said plurality of eukaryotic cells with a ligand of said protein of interest,

- 5 determining expression from said β -lactamase expression constructs, and identifying plurality of said polynucleotides exhibiting a increase, decrease or no change in expression from said β -lactamase expression that results from either said expressing of said protein of interest or said contacting of said ligand,

10 wherein an increase, decrease or no change in expression of each said polynucleotide from said plurality of polynucleotides indicates a profile of cellular response relating to said protein of interest.

115. A method for determining a cellular response profile for a chemical, comprising:
 expressing a protein of interest in a plurality of eukaryotic cells, wherein each eukaryotic cell comprises a genomic polynucleotide with a β -lactamase expression construct under expression control by a polynucleotide in said genomic polynucleotide, and said plurality of cells has a plurality of integration sites where said β -lactamase expression construct has integrated into said genome of each said eukaryotic cell,
 optionally contacting said plurality of eukaryotic cells with a ligand of said protein of interest,

- 20 contacting said plurality of eukaryotic cells with a test chemical at a predetermined concentration, and
 determining expression from said β -lactamase expression constructs, and identifying plurality of said polynucleotides exhibiting a increase, decrease or no change in expression from said β -lactamase expression that results from either said expressing of said protein of interest or said contacting of said ligand,

25 wherein an increase, decrease or no change in expression of each said polynucleotide from said plurality of polynucleotides indicates a profile of cellular response relating to said test chemical.

116. A method for identifying a modulator of a viral component, comprising:

contacting a eukaryotic cell with a test chemical at a predetermined concentration, wherein said eukaryotic cell comprises 1) a genomic polynucleotide with a β -lactamase expression construct under expression control by a first polynucleotide in said genomic polynucleotide and 2) a viral component is not previously known to modulate transcription of a gene product under expression control of said first polynucleotide and said viral component is not an oncogene or proto-oncogene or protein product thereof, and

determining expression of said β -lactamase expression construct.

117. The method of claim 116, wherein said viral component is selected from the list consisting of a virus, a capsule, a viral polynucleotide, or a viral protein.

118. The method of claim 117, further comprising contacting a second eukaryotic cell with said test chemical at a predetermined concentration, wherein said eukaryotic cell comprises 1) a second genomic polynucleotide with a β -lactamase expression construct under expression control by a second polynucleotide in said second genomic polynucleotide and 2) said viral component, and

determining expression of said β -lactamase expression construct, wherein said viral component is selected from the list consisting of a virus, a capsule, a viral polynucleotide, or a viral protein.

119. The method of claim 118, wherein said second eukaryotic cell is from a population of eukaryotic cells, each said eukaryotic cell comprising 1) a genomic polynucleotide with a β -lactamase expression construct and 2) said viral component.

120. A method for identifying a cellular function of a viral component, comprising:
contacting a eukaryotic cell with a viral component at a predetermined concentration or expressing a viral component in said eukaryotic cell, wherein said eukaryotic cell comprises 1) a genomic polynucleotide with a β -lactamase expression construct under expression control by a first polynucleotide in said genomic polynucleotide,

optionally contacting said eucaryotic cell with a second viral component of a virus that is different from said virual component,

determining expression of said β -lactamase expression construct, and identifying the function of said genomic polynucleotide with said β -lactamase expression construct or gene where said β -lactamase expression construct has integrated.

121. A method for identifying a chemical that modulates a physiological response or cellular pathway, comprising:

contacting a eukaryotic cell with a test chemical at a predetermined concentration, wherein said eukaryotic cell comprises 1) a genomic polynucleotide with a β -lactamase expression construct under expression control by a first polynucleotide in said genomic polynucleotide, wherein said cell is characterized as comprising a physiological response of interest or a cellular pathway of interest, and

10 contacting said eukaryotic cell with a signal molecule, and determining expression of said β -lactamase expression construct.

122. The method of claim 121, said signal molecule is a naturally occurring molecule that binds to the outside of said eukaryotic cell and said eukaryotic cell is a mammalian cell.

123. The method of claim 122, said physiological response occurs in vivo in an cell selected from the group consisting of a nerve cell, cardiac cell, epithelial cell, muscle cell, endocrine cell, paracrine cell, blood cell, and connective tissue cell.

124. The method of claim 121, wherein said signal molecule increases expression.
125. The method of claim 124, wherein said polynucleotide has a gene product that does not alter said cellular pathway or physiological response.

126. A chemical identified by any of the above methods for identifying useful chemicals.

127. A method for identifying and developing a drug, comprising:
- 25 1) contacting a population of non-yeast, eukaryotic cells with a test chemical and a known modulator, wherein said population of non-yeast, eukaryotic cells comprises a genome with a stably integrated β -lactamase expression construct, comprising:
- a) a polynucleotide encoding a protein having β -lactamase activity, and

- b) a splice acceptor site; and
- 2) detecting expression of said β -lactamase polynucleotide expressed by said population of non-yeast, eukaryotic cells, wherein said known modulator increases or decreases the expression of said polynucleotide encoding a protein having β -lactamase activity, and said known modulator modulates a biological process or target,
- 5 3) determining whether said test chemical alters expression of said β -lactamase polynucleotide,
- 4) optionally testing for toxic effects of said test chemical in a cell-based assay,
- 10 5) optionally generating a second test chemical based on the structure-property relationships of said test chemical,
- 6) optionally determining whether said second test chemical alters expression of said β -lactamase polynucleotide,
- 15 7) testing for toxic effects of said test chemical or said second test chemical in a mammal, and
- 8) testing for therapeutic effects of said test chemical or said second test chemical in a mammal.
128. A drug chemical identified and developed by the following method, comprising:
- 1) contacting a population of non-yeast, eukaryotic cells with a test chemical and 20 a known modulator, wherein said population of non-yeast, eukaryotic cells comprises a genome with a stably integrated β -lactamase expression construct, comprising:
- a) a polynucleotide encoding a protein having β -lactamase activity, and
- b) a splice acceptor site; and
- 25 2) detecting expression of said β -lactamase polynucleotide expressed by said population of non-yeast, eukaryotic cells, wherein said known modulator increases or decreases the expression of said polynucleotide encoding a protein having β -lactamase activity, and said known modulator modulates a biological process or target,

- 3) determining whether said test chemical alters expression of said β -lactamase polynucleotide,
- 4) optionally testing for toxic effects of said test chemical in a cell-based assay,
- 5) optionally generating a second test chemical based on the structure-property relationships of said test chemical,
- 6) optionally determining whether said second test chemical alters expression of said β -lactamase polynucleotide,
- 7) testing for toxic effects of said test chemical or said second test chemical in a mammal, and
- 10 8) testing for therapeutic effects of said test chemical or said second test chemical in a mammal.

129. The drug of claim 128, wherein said drug can be used to treat a medical condition selected from the group consisting of immune response, cardiac disfunctions and disease, vascular disfunctions and diseases, neural disfunctions and disease, endocrine disfunctions and disease, gastro-intestinal disfunctions and disease, obesity, diabetes, inflammation disfunctions and disease, cancer and trauma.

130. A pharmaceutical composition, comprising a therapeutic agent and a pharmaceutically acceptable carrier.

131. The pharmaceutical composition of claim 130, said therapeutic agent having the structure of Chemical A or B and said pharmaceutically acceptable carrier is selected for treating undesired T-cell activation or an undesired immune response.

1/4

**Application of a Prior Art
Reporter Gene with Methods
Described Herein**

One Embodiment of the Invention

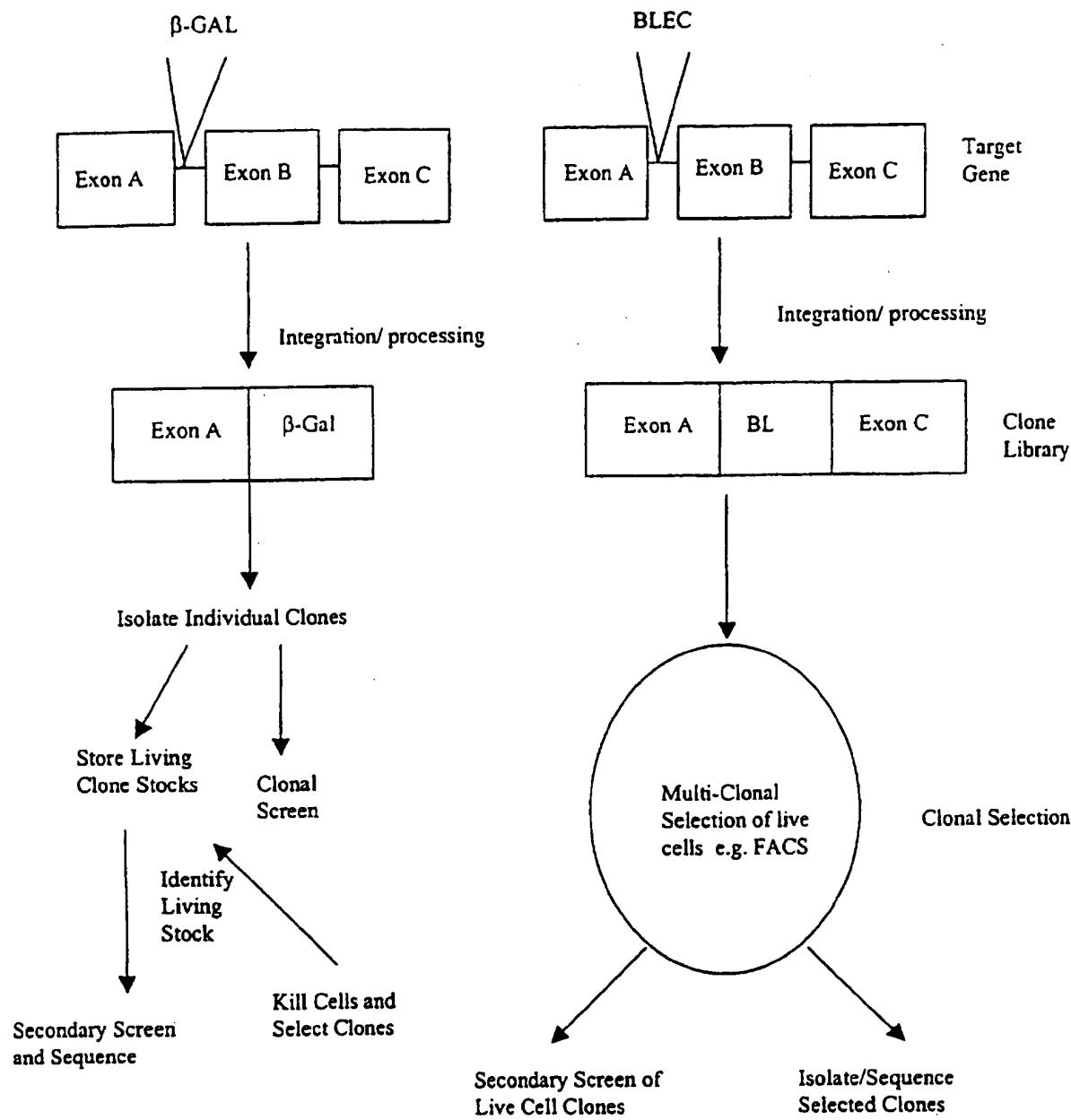


FIG. 1

Genomic Assay System (GAS)

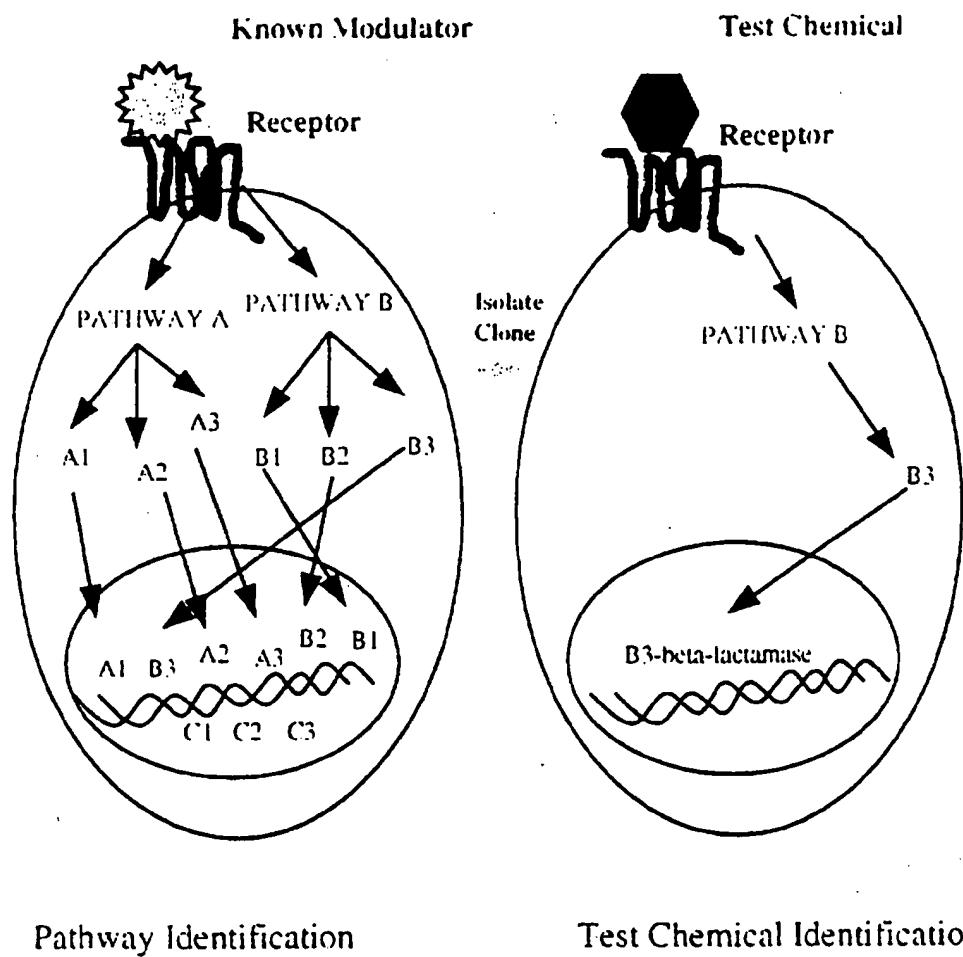
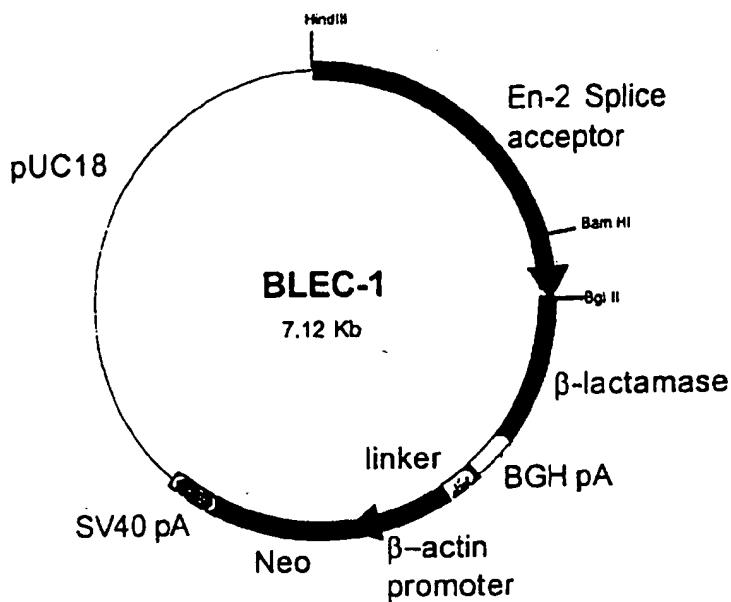


FIG. 2

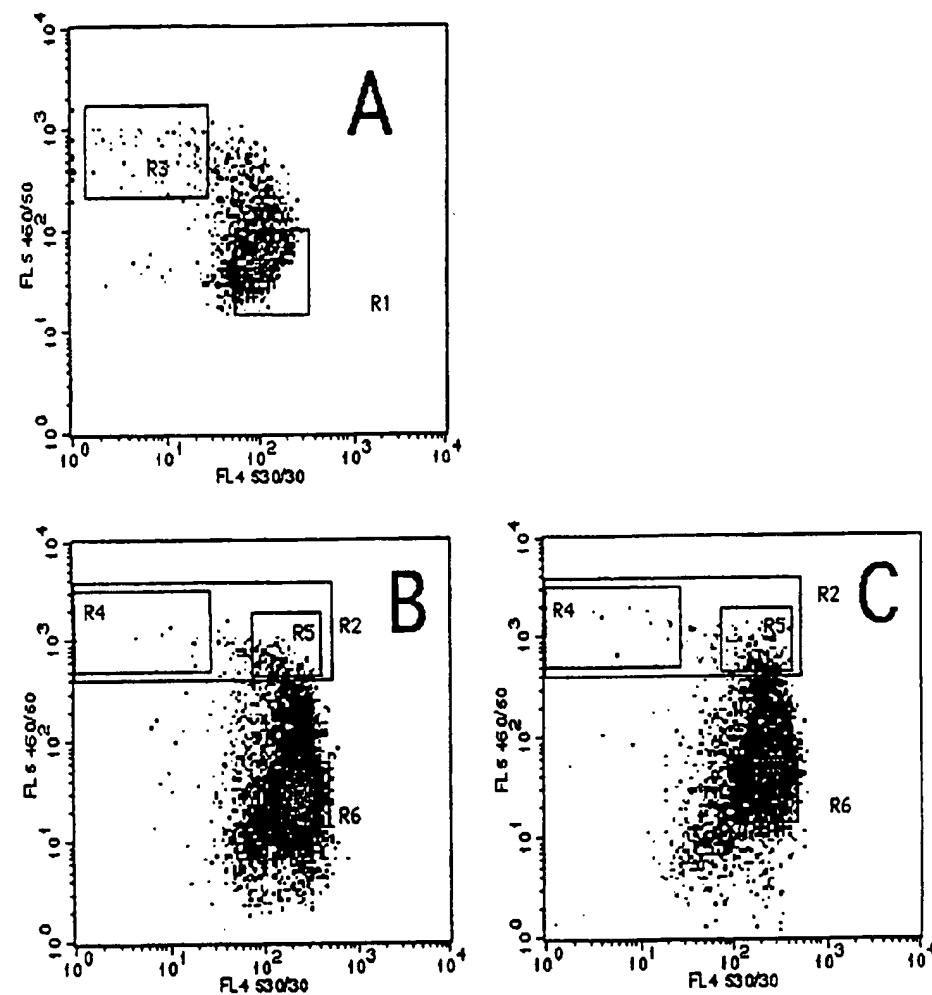


5

10

15

FIG. 3



5

10

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17395

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,298,429 A (EVANS et al.) 29 March 1994, see entire document.	1-125, 127-129
Y	US 5,514,561 (QUANTE et al.) 07 May 1996, see entire document.	1-125, 127-129
Y	WO 94/24301 (THE UNIVERSITY OF EDINBURGH) 27 October 1994, see entire document.	1-125, 127-129
Y,P	WO 96/30540 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 03 October 1996, see entire document.	1-125, 127-129
X	TADROS et al. Synthesis of quinoline derivatives. Indian Journal of Chemistry. June 1976, Vol 14B, pages 467-469, especially page 468, column 1.	128-131

 Further documents are listed in the continuation of Box C.

See patent family annex.

• Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
• "A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
• "B" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
• "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A"	document member of the same patent family
• "O" document referring to an oral disclosure, use, exhibition or other means		
• "P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

16 JANUARY 1998

Date of mailing of the international search report

10 FEB 1998

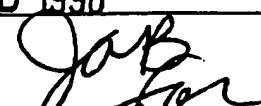
Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JEFFREY FREDMAN

Telephone No. (703) 308-0196



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17395

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SKARNES et al. A gene trap approach in mouse embryonic stem cells: the lacZ reporter is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice. Genes and Development. 1992, Vol. 6, pages 903-918, see entire document.	1-125, 127-129
Y	REDDY et al. Fluorescence-activated sorting of totipotent embryonic stem cells expressing developmentally regulated lacZ fusion genes. Proc. Natl. Acad. Sci. USA. August 1992, Vol. 89, pages 6721-6725, see entire document.	1-125, 127-129
Y	KUSPA et al. Tagging developmental genes in Dictyostelium by restriction enzyme-mediated integration of plasmid DNA. Proc. Natl. Acad. Sci. USA. 1992, Vol.89, pages 8803-8807, see entire document.	1-125, 127-129
Y	REID et al. Cotransformation and gene targeting in Mouse embryonic stem cells. Molecular and Cellular Biology. May 1991, Vol. 11, No. 5, pages 2769-2777, see entire document.	1-125, 127-129

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US97/17395**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: 126
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17395

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07D 221/02, 215/12; C12Q 1/70, 1/68, 1/08; C12P 21/06; C12N 15/00, 9/14, 9/84, 9/86; G01N 33/53; C07H 21/02, 21/04; A01N 43/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

S46/112, 174; 435/5, 6, 40.51, 69.1, 69.8, 70.1, 91.1, 172.1, 172.3, 195, 230, 231, 325, 968; 536/23.1, 24.3, 24.31, 24.5; S14/44

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

S46/112, 174; 435/5, 6, 40.51, 69.1, 69.8, 70.1, 91.1, 172.1, 172.3, 195, 230, 231, 325, 968; 536/23.1, 24.3, 24.31, 24.5; S14/44

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN REGISTRY, BEILSTEIN ONLINE, BIOSIS, CJACS, DRUGU, EMBASE, EUROPATFULL, MEDLINE, PATOSWO, SCISEARCH, TOXSLIT
search terms: lactamase, reporter, marker, label, fluorescein, gene, trap, ligand, assay, screening, method, vivo, vitro

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-125 and 127, drawn to methods for identifying proteins or chemicals/drugs employing beta-lactamase constructs.

Group II, claim(s) 128-131, drawn to chemicals/drugs identified by screening with beta-lactamase constructs.

Note that claim 126 is not listed. It is an improper multiple dependent claim that fails to claim the multiple embodiments in the alternative (PCT Rule 6.4) and is thus withdrawn under nonestablishment. If it were included it would be listed with Group II.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Applicant is required to elect one species of chemical/drug encompassed by claims 128-131 of Group II selected from modulators of the following:

- hormone receptors
- intracellular receptors
- receptors of the cytokine super family
- G-coupled protein receptors
- heterologous G-proteins
- neurotransmitter receptors
- tyrosine kinase receptors
- viral components
- physiological responses
- cellular pathways
- chemical A
- chemical B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17395

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The chemicals/drugs that are identifiable by the methods of Group I include admittedly old chemicals (description pages 66+). More characterization of old chemicals as being active in the assays of Group I cannot impart novelty over the prior art to the chemicals themselves. The claimed invention of Group II thus lacks a special technical feature that defines a contribution over the prior art.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: The vast number of chemicals/drugs encompassed by Group II include numerous already known chemicals. These have no common core structure and no common property or activity; further they do not belong to any recognized class of chemical compounds in the art to which the invention pertains.